PATENT APPLICATION

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TITLE: **ANTIBODY COMPLEXES AND METHODS FOR IMMUNOLABELING**

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ANTIBODY COMPLEXES AND METHODS FOR IMMUNOLABELING

INTRODUCTION

Cross-Reference to Related Applications

This application claims priority to US Serial No. 60/329,068, filed October 12, 2001; US Serial No. 60/369,418 filed April 1, 2002, US Serial No. 10/118,204 filed April 5, 2002, and PCT/US02/31416 filed October 2, 2002, which disclosures are herein incorporated by reference.

Field of the Invention

The present invention relates to immuno-labeled complexes and methods for use in the detection and measurement of one or more targets in a biological sample. The invention has applications in the fields of molecular biology, cell biology, immunohistochemistry, diagnostics, and therapeutics.

Background of the Invention

Immunolabeling is a method for qualitative or quantitative determination of the presence of a target in a sample, wherein antibodies are utilized for their specific binding capacity. The antibodies form a complex with the target (antigen), wherein a detectable label is present on the antibody or on a secondary antibody. The detectable label is a key feature of immunolabeling, which can be detected directly or indirectly. The label provides a measurable signal by which the binding reaction is monitored providing a qualitative and/or quantitative measure of the degree of binding. The relative quantity and location of signal generated by the labeled antibodies can serve to indicate the location and/or concentration of the target. The label can also be used to select and isolate labeled targets, such as by flow sorting or using magnetic separation media. Examples of labels include but are not limited to radioactive nucleotides (^{125}I, ^{3}H, ^{14}C, ^{32}P), chemiluminescent, fluorescent, or phosphorescent compounds (e.g., dioxetanes, xanthene, or carbocyanine dyes, lanthanide chelates), particles (e.g., gold clusters, colloidal gold, microspheres, quantum dots), and enzymes (e.g., peroxidases, glycosidases, phosphatases, kinases). Ideally, the label is attached to the antibody in a manner that does not perturb the antibody's binding characteristics but enables the label to be measured by an appropriate detection technology. The choice of labels is influenced by factors such as ease and sensitivity of detection, equipment availability, background in the sample (including other labels) and the degree to

which such labels are readily attached to the particular antibody. Both direct and indirect labeling of antibodies is utilized for immunolabeling. Direct labeling utilizes only a primary antibody, i.e. the antibody specific for the target, bound to the label. In contrast, indirect labeling utilizes a secondary antibody bound to the label, which is specific for the primary antibody, e.g. a goat anti-rabbit antibody. The principal differences in immunolabeling methods and materials reside in the way that the label is attached to the antibody–antigen complex, the type of label that is used, and the means by which the antibody–antigen complex is detected.

Limitations for direct labeling primary antibodies include the need for buffers free of primary amines, or carrier proteins such as bovine serum albumin (BSA), and other compounds such as tris-(hydroxymethyl)aminomethane (TRIS), glycine, and ammonium ions. These materials are, however, common components in antibody buffers and purification methods, and it may not be possible or feasible to remove them prior to the coupling reaction. In particular, many monoclonal antibodies are available only as ascites fluid or in hybridoma culture supernatants, or diluted with carrier proteins, such as albumins. Thus, direct labeling of antibodies in ascites fluid or other medias containing interfering compounds is not attainable.

The indirect immunolabeling method typically involves a multi-step process in which an unlabeled first antibody (typically a primary antibody) is directly added to the sample to form a complex with the antigen in the sample. Subsequently, a labeled secondary antibody, specific for the primary antibody, is added to the sample, where it attaches noncovalently to the primary antibody–antigen complex. Alternatively, a detectable label is covalently attached to an immunoglobulin-binding protein such as protein A and protein G to detect the antibody–antigen complex that has previously been formed with the target in the sample. Using ligands, such as streptavidin, that are meant to amplify the detectable signal also expands this cascade binding.

Indirect immunolabeling often results in false positives and high background. This is due to the fact that secondary antibodies, even when purified by adsorption against related species, nevertheless can exhibit significant residual cross-reactivity when used in the same sample. For example, when mouse tissue is probed with a mouse monoclonal antibody, the secondary antibody must necessarily be a labeled anti-mouse antibody. This anti-mouse antibody will detect the antibody of interest but will inevitably and additionally detect irrelevant, endogenous mouse immunoglobulins inherent in mouse tissue. This causes a significant background problem, especially in diseased tissues, which reduces the

usefulness and sensitivity of the assay. Thus, the simultaneous detection of more than one primary antibody in a sample without this significant background interference depends on the availability of secondary antibodies that 1) do not cross-react with proteins intrinsic to the sample being examined, 2) recognize only one of the primary antibodies, and 3) do not recognize each other (Brelje, et al., METHODS IN CELL BIOLOGY 38, 97-181, especially 111-118 (1993)).

To address the background problem in indirect labeling, a number of strategies have been developed to block access of the anti-mouse secondary antibodies to the endogenous mouse immunoglobulins. One such strategy for blocking involves complexing the primary antibody with a selected biotinylated secondary antibody to produce a complex of the primary and secondary antibodies, which is then mixed with diluted normal murine serum (Trojanowski et al., U.S. Pat No. 5,281,521 (1994)). This method is limited by the necessity to utilize an appropriate ratio of primary–secondary complex. Too low a ratio of primary–secondary complex will cause a decrease in specific staining and increased background levels due to the uncomplexed secondary anti-mouse antibody binding to endogenous mouse antibodies. However, the ability of a whole IgG antibody (as was used in the referenced method) to simultaneously bind and cross-link two antigens results in too high a ratio, causing the complex to precipitate or form complexes that are too large to penetrate into the cell or tissue.

Another strategy for blocking access to endogenous immunoglobulins in the sample involves pre-incubating the sample with a monovalent antibody, such as Fab' fragments, from an irrelevant species that recognize endogenous immunoglobulins. This approach requires large quantities of expensive Fab' fragments and gives mixed results and adds at least two steps (block and wash) to the overall staining procedure. The addition of a cross-linking reagent has resulted in improved reduction of background levels (Tsao, et al., U.S. Pat. No. 5,869,274 (1997)) but this is problematic when used with fluorophore-labeled antibodies. The cross-linking causes an increase in the levels of autofluorescence and thus the background (J. Neurosci. Meth. 83, 97 (1998); Mosiman et al., Methods 77, 191 (1997); Commun. Clin. Cytometry 30, 151 (1997); Beisker et al., Cytometry 8, 235 (1987)). In addition, pre-incubation with a cross-linking reagent often masks or prevents the antibody from binding to its antigen (J. Histochem. Cytochem. 45, 327 (1997); J. Histochem. Cytochem. 39, 741 (1991); J. Histochem. Cytochem. 43, 193 (1995); Appl. Immunohistochem. Molecul. Morphol. 9, 176 (2001)).

In a variation of this blocking strategy, a multi-step sequential-labeling procedure is used to overcome the problems of cross-reactivity. The sample is incubated with a first antibody to form a complex with the first antigen, followed by incubation of the sample with a fluorophore-labeled goat Fab anti-mouse IgG to label the first antibody and block it from subsequently complexing when the second antibody is added. In the third step, a second mouse antibody forms a complex with the second antigen. Because the second antibody is blocked from cross-reacting with the first antibody, the second mouse antibody is detected with a standard indirect-labeling method using a goat anti-mouse antibody conjugated to a different fluorescent dye (J. Histochem. Cytochem. 34, 703 (1986)). This process requires multiple incubation steps and washing steps and it still cannot be used with mouse antibodies to probe mouse tissue.

Another blocking method is disclosed in the animal research kit (ARK) developed by DAKO. In this kit, a primary antibody is complexed with biotin-labeled goat Fab anti–mouse IgG and excess free Fab is blocked with normal mouse serum. However, since the Fab used in this process is generated from the intact IgG (rather than a selected region) there is a potential for the formation of anti-paratope or anti-idiotype antibodies that will block the antigen-binding site and prevent immunolabeling. The biotinylated antibody also requires subsequent addition of a labeled avidin or streptavidin conjugate for its subsequent visualization.

The present invention is advantageous over previously described methods and compositions in that it provides the benefits of indirect labeling with the easy and flexibility of direct labeling for determination of a desired target in a biological sample. The present invention provides labeled monovalent proteins specific for a target-binding antibody, which are complexed prior to addition with a biological sample. Because these monovalent proteins are not bivalent antibodies, precipitation and cross-linking are not a problem. Therefore the compositions of the present invention can be used with immunologically similar monoclonal or polyclonal antibodies of either an identical isotype or different isotypes. The monovalent labeling reagents are specific for the Fc region of target-binding antibodies, these reagents will not interfere with the binding region of the primary antibody. In addition, the monovalent labeling reagents are not negatively affected by the presence of primary amines like BSA, gelatin, hybridoma culture supernatants or ascites fluid, thus primary antibodies present in these media can be effectively labeled with the labeling reagents of the present invention. Thus, the present invention provides numerous advantages over the conventional methods of immunolabeling.

SUMMARY OF THE INVENTION

The present invention provides labeling reagents and methods for labeling primary antibodies and for detecting a target in a sample using an immuno-labeled complex that comprises a target-binding antibody and one or more labeling reagents. The labeling reagents comprise monovalent antibody fragments or non-antibody monomeric proteins whereby the labeling reagents have affinity for a specific region of the target-binding antibody and are covalently attached to a label. Typically, the labeling reagent is an anti-Fc Fab or Fab' fragment that was generated by immunizing a goat or rabbit with the Fc fragment of an antibody.

The methods for labeling a target-binding antibody with a labeling reagent comprise a) contacting a solution of target-binding antibodies with a labeling reagent, b) incubating said target-binding antibodies and said labeling reagent wherein a region of said target binding antibody is selectively bound by labeling reagent, and c) optionally removing unbound labeling reagent by adding a capture reagent comprising immunoglobulin proteins or fragments thereof that are optionally immobilized on a matrix. The labeling of the target-binding antibody can be performed irrespective of the solution that the antibody is present in and includes proteins that are normally present in serum or ascites. This feature of the labeling process of the target-binding antibody eliminates the need to purify and concentrate the target-binding antibody. The time required for the labeling reagent to selectively bind to the target-binding antibody is typically very short, often less than 10 minutes. Often the labeling reagent binds the target-binding antibody in the amount of time it takes to add and mix the labeling reagent with the target-binding antibody. This formation of an immuno-labeled complex – a target-binding antibody and a labeling reagent – results in the formation of an target detection solution that is used to detect a target in a sample.

The labeling steps of the target-binding antibody are optionally repeated to form a panel of subsets, these immuno-labeled complex subsets may be used individually or pooled wherein each subset is distinguished from another subset by i) the target-binding antibody, or ii) a ratio of label to labeling reagent, or iii) a ratio of labeling reagent to the target-binding antibody or iv) by a physical property of the label. Thus, it is appreciated that a wide range of subsets can be formed wherein the subsets can be used individually to detect a target in a sample or pooled to simultaneously detect multiple targets in a sample. The simultaneous detection of multiple targets in a sample is especially useful in methods that utilize flow cytometry or methods that immobilize a population of cells or tissue on a surface.

The methods for determining a target in a sample using immuno-labeled subsets comprises forming a subset of immuno-labeled complexes, as described above, contacting a sample with said immuno-labeled complexes, incubating the sample for a time sufficient to allow the immuno-labeled complex to selectively bind to a desired target, and illuminating the immuno-labeled complex whereby the target is detected. The sample is any material that may contain a target and typically comprises a population of cells, cellular extract, subcellular component, proteins, peptides, tissue culture, tissue, a bodily fluid, or a portion or combination thereof. When multiple targets are detected a pooled subset of immuno-labeled complexes are formed and incubated with the sample or individual subsets are add sequentially to a sample. For methods using flow cytometry the population of cells is illuminated when they pass through an optical examination zone and the data collected about the label determines the identity and quantity of the targets.

In addition the labeling reagents are used to determine the presence of a target ligand in a sample employing a target-binding antibody and a ligand analog to form a ligand-detection reagent. The ligand-detection reagent comprises a ligand-binding antibody, a ligand analog to form an antibody-ligand analog complex wherein the ligand analog is covalently bonded to a reporter molecule and a labeling reagent non-covalently bonded to a region of the antibody to form a ternary complex. The labeling reagent comprises a monovalent antibody fragment or a non-antibody protein that is covalently bonded to a label moiety. The reporter molecule, when forming part of the ligand-detection reagent is quenched by the label moiety of the labeling reagent, wherein the amount of quenching is directly related to the amount of ligand present in the sample. Thus, the target ligand displaces the ligand analog relieving quenching of the reporter molecule. Alternatively, the ligand analog is fluorogenic wherein the ligand analog is essentially non-fluorescent in solution but when bound by the ligand-binding antibody the detectable signal increases. In this instance a decrease in signal, as opposed to the relieving of quenching, is measured for the presence of a target ligand.

The ligand analog is covalently attached to a reporter molecule selected from the group consisting of a borapolyazaindacene, a coumarin, a xanthene, a cyanine, a fluorescent protein and a phosphorescent dye. Through careful selection of the reporter molecule, ligand analog, ligand-binding antibody and labeling reagent we have demonstrated that the reporter molecule can be substantially quenched or masked when bound by the ligand-binding antibody. In this instance, the labeling reagent is covalently attached to a label wherein the label is capable of absorbing energy from the reporter molecule to form an energy transfer pair when complexed with the ligand-binding antibody. The emitted energy

from the reporter molecule is either absorbed by the label and re-emitted at a longer wavelength than energy emitted by the reporter molecule or is absorbed with little or no energy being re-emitted at a longer wavelength. In this way the label is either considered a quencher or a fluorophore moiety, both of which are capable of absorbing energy.

Therefore, a method is provided for determining the presence of a ligand in a sample employing the ligand-detection reagent. In carrying out the present methods the ligand analog-reporter molecule and labeling reagent is complexed with the ligand-binding antibody wherein the reporter molecule is quenched or masked. The ligand-detection reagent is incubated with the sample for a sufficient amount of time to allow for the target ligand present in the sample to displace the ligand-analog. In this way the unmasking of the reporter molecule provides either the presence of a detectable signal or a shift in color compared to when the ligand-binding antibody bound the ligand analog.

The present invention provides novel labeling reagents, ligand-detection reagents, ligand-analogs and a competitive immunoassay for the determination of the presence of a target ligand in a sample.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Shows a schematic representation of the formation of the immuno-labeled complex (target-binding antibody and labeling reagent).

Figure 2: Shows species specificity of goat Fab anti–(mouse Fc), as observed using a microplate coated with IgG of various species. The various species were blocked with BSA, reacted with biotinylated goat Fab anti–(mouse Fc), washed, and then treated with streptavidin–horseradish peroxidase (HRP), followed by hydrogen peroxide (H_2O_2) and the Amplex Red peroxidase detection reagent.

Figure 3: Shows a preferred molar ratio of a goat Fab anti–(mouse Fc) labeling reagent. Varying amounts of an Alexa Fluor 488 dye–labeled Fab fragment of goat anti–(mouse Fc) were added to a constant amount of anti-biotin monoclonal antibody (mAb). This mixture was equilibrated for 20 minutes, and then added to biotinylated-BSA in a microplate well. After allowing time to bind, the plates were washed and the remaining fluorescence was quantitated. The analysis was performed in triplicate (circles). Control experiments were

performed, as described above, but without adding the primary anti-biotin antibody (solid squares).

Figure 4: Shows a comparison of the fluorescence intensity (Example 6) for labeling reagent prepared in homogeneous solution (Example 4) and labeling reagent prepared on a column (Example 5).

Figure 5: Shows detection of multiple targets on T cells using a labeling reagent attached to a R-phycoerythrin (R-PE) (Fig. 5A) to detect CD3-positive T cells, a labeling reagent attached to Alexa Fluor 647 dye (Fig. 5B) to detect CD4-positive T cells and a labeling reagent attached to Alexa Fluor 488 dye (Fig 5B) to detect CD8-positive T cells (**Example 18**). The CD-3 detected T cells are shown in the upper left (UL) and upper right (UR) quadrants. The relative percentages of total lymphocytes that are CD3-positive cells are 83.3% (UL+UR). The relative percentage of CD8-positive Alexa Fluor 488 dye–stained lymphocytes and CD3-positive R-PE dye–stained lymphocytes is 35.1% (UR quadrant). The lower left quadrant (LL, 20.4%) shows CD3-negative lymphocytes (i.e. non–T cells) comprised of NK cells, B cells and some monocytes. In the lower right (LR, 2.7%) region are non–T cells, which are nonspecifically stained. Figure 5B further shows CD3-positive T-cells subdivided into Alexa Fluor 647 dye CD4-positive and Alexa Fluor 488 dye CD8-positive. CD4-positive cells represent 50.9% of total lymphocytes (UL quadrant) and CD8-positive cells represent 24.5% of the total lymphocytes (LR quadrant). The 23.1% of cells in the LL quadrant are non-T cells, while the 1.5% of cells in UR quadrant are likely nonspecifically stained lymphocytes.

Figure 6: Shows high-performance size-exclusion chromatographic analysis of Alexa Fluor 488 dye–labeled goat Fab anti–(mouse Fc) labeling reagent binding to a mouse IgG_1 target-binding antibody. The labeling reagent, alone, appears as a peak at 38 minutes; the target-binding antibody, alone, appears as a peak at 33 minutes. When labeling reagent and target-binding antibody are mixed together at a molar ratio of ~5:1 (labeling reagent:target-binding antibody), the resulting immunolabeling complex appears as a peak at 29 minutes (**Example 10**).

Figure 7: Shows the production of labeling reagent wherein the label is attached to the labeling reagent when immobilized on a column.

Figure 8: Shows a schematic representation of the ligand-detection reagent comprising a ligand analog, ligand-binding antibody and labeling reagent.

Figure 9: Shows the amount of fluorescence quenching by BODIPY-FL Digoxigenin ligand analog when bound to the ligand-binding antibody/Fab fragment of goat anti-mouse kappa chain conjugated to QSY-9 complex. As the amount of anti Digoxigenin/Fab fragment increases, the fluorescence of the BODIPY-FL Digoxigenin decreases. When target ligand (Digoxigenin) is added the fluorescence quenching is partially relieved.

Figure 10: Shows the use of a fluorogenic ligand analog (Compound **4**) that upon interaction with the ligand-binding antibody exhibits fluorescence enhancement.

Figure 11: Shows the quenching of the ligand analog (Compound **15**) when bound by the ligand-binding antibody and the subsequent relief of quenching when target ligand, phosphotyrosine peptides, are added and the ligand analog displaced from the binding groove of the phosphotyrosine ligand-binding antibody.

Figure 12: Shows the off rate of the ligand analog (Compound **15**) when target ligand is added to the ligand-detection reagent.

Figure 13: Shows the selectivity of the ligand-binding antibody for the target ligand and ligand analog (Compound **15**)

Figure 14: Shows that the use of ATP in a kinase assay does not compete for binding of the phosphotyrosine ligand-binding antibody.

Figure 15: Shows the detection of Abl kinase activity using Compound **15** as the ligand analog, phosphotyrosine ligand-binding antibody as the ligand-binding antibody and MPIJ-5 as the kinase substrate and subsequent target ligand.

Figure 16: Shows the ability of the ligand-detection reagent to detect the presence of an inhibitor of kinase activity (staurosporine).

Figure 17: Shows the screening of multiple phosphotyrosine ligand-binding antibodies to optimize the affinity of the ligand analog (Compound **16**) for the ligand-binding antibody.

Figure 18: Shows a method for selecting ligands for use in displacement assays. Figure 14A shows the measure of fluorescence polarization as a function of antibody concentration, figure 14B shows the measure of fluorescence intensity as a function of antibody concentration, figure 14C shows a measure of fluorescence enhancement in the presence of target ligand (phosphopeptide) and figure 14D shows the confirmation of fluorescence enhancement by depolarization of the ligand analog.

Figure 19: Shows the ability of the labeling reagent to quench the reporter molecule when present as a ligand detection reagent ternary complex.

DETAILED DESCRIPTION OF THE INVENTION

I. DEFINITIONS

Before describing the present invention in detail, it is to be understood that this invention is not limited to specific compositions or process steps, as such may vary. It should be noted that, as used in this specification and the appended claims, the singular form "a", "an" and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a protein labeling complex" includes a plurality of complexes and reference to "a target-binding protein" includes a plurality of proteins and the like.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention is related. The following terms are defined for purposes of the invention as described herein.

The term "affinity" as used herein refers to the strength of the binding interaction of two molecules, such as an antibody and an antigen or a positively charged moiety and a negatively charged moiety. For bivalent molecules such as antibodies, affinity is typically defined as the binding strength of one binding domain for the antigen, e.g. one Fab fragment for the antigen. The binding strength of both binding domains together for the antigen is referred to as "avidity". As used herein "High affinity" refers to a ligand that binds to an antibody having an affinity constant (K_a) greater than 10^4 M^{-1}, typically 10^5–10^{11} M^{-1}; as determined by inhibition ELISA or an equivalent affinity determined by comparable

techniques such as, for example, Scatchard plots or using K_d/dissociation constant, which is the reciprocal of the K_a, etc.

The term "antibody" as used herein refers to a protein of the immunoglobulin (Ig) superfamily that binds noncovalently to certain substances (e.g. antigens and immunogens) to form an antibody–antigen complex. Antibodies can be endogenous, or polyclonal wherein an animal is immunized to elicit a polyclonal antibody response or by recombinant methods resulting in monoclonal antibodies produced from hybridoma cells or other cell lines. It is understood that the term "antibody" as used herein includes within its scope any of the various classes or sub-classes of immunoglobulin derived from any of the animals conventionally used.

The term "antibody fragments" as used herein refers to fragments of antibodies that retain the principal selective binding characteristics of the whole antibody. Particular fragments are well-known in the art, for example, Fab, Fab', and $F(ab')_2$, which are obtained by digestion with various proteases, pepsin or papain, and which lack the Fc fragment of an intact antibody or the so-called "half-molecule" fragments obtained by reductive cleavage of the disulfide bonds connecting the heavy chain components in the intact antibody. Such fragments also include isolated fragments consisting of the light-chain-variable region, "Fv" fragments consisting of the variable regions of the heavy and light chains, and recombinant single chain polypeptide molecules in which light and heavy variable regions are connected by a peptide linker. Other examples of binding fragments include (i) the Fd fragment, consisting of the VH and CH1 domains; (ii) the dAb fragment (Ward, et al., Nature 341, 544 (1989)), which consists of a VH domain; (iii) isolated CDR regions; and (iv) single-chain Fv molecules (scFv) described above. In addition, arbitrary fragments can be made using recombinant technology that retains antigen-recognition characteristics.

The term "antigen" as used herein refers to a molecule that induces, or is capable of inducing, the formation of an antibody or to which an antibody binds selectively, including but not limited to a biological material. Antigen also refers to "immunogen". The target-binding antibodies selectively bind an antigen, as such the term can be used herein interchangeably with the term "target".

The term "anti-region antibody" as used herein refers to an antibody that was produced by immunizing an animal with a select region that is a fragment of a foreign antibody wherein only the fragment is used as the immunogen. Anti-region antibodies include monoclonal and polyclonal antibodies. The term "anti-region fragment" as used herein refers to a

monovalent fragment that was generated from an anti-region antibody of the present invention by enzymatic cleavage.

The term "biotin" as used herein refers to any biotin derivative, including without limitation, substituted and unsubstituted biotin, and analogs and derivatives thereof, as well as substituted and unsubstituted derivatives of caproylamidobiotin, biocytin, desthiobiotin, desthiobiocytin, iminobiotin, and biotin sulfone.

The term "biotin-binding protein" as used herein refers to any protein that binds selectively and with high affinity to biotin, including without limitation, substituted or unsubstituted avidin, and analogs and derivatives thereof, as well as substituted and unsubstituted derivatives of streptavidin, ferritin avidin, nitroavidin, nitrostreptavidin, and Neutravidin™ avidin (a de-glycosylated modified avidin having an isoelectric point near neutral).

The term "buffer" as used herein refers to a system that acts to minimize the change in acidity or basicity of the solution against addition or depletion of chemical substances.

The term "capture reagent" refers to a non-specific immunoglobulin that is used to remove excess labeling reagent after the formation of the immuno-labeled complex. The capture reagent is optionally attached a matrix to facilitate removal of the excess labeling regent. A matrix typically includes a microsphere, an agarose bead or any solid surface that the excess labeling reagent can be passed by.

The term "chromophore" as used herein refers to a label that emits light in the visible spectra that can be observed without the aid of instrumentation.

The term "complex" as used herein refers to the association of two or more molecules, usually by non-covalent bonding, e.g., the association between an antibody and an antigen or the labeling reagent and the target-binding antibody.

The term "detectable response" as used herein refers to an occurrence of, or a change in, a signal that is directly or indirectly detectable either by observation or by instrumentation. Typically, the detectable response is an occurrence of a signal wherein the fluorophore is inherently fluorescent and does not produce a change in signal upon binding to a metal ion or biological compound. Alternatively, the detectable response is an optical response resulting in a change in the wavelength distribution patterns or intensity of absorbance or fluorescence or a change in light scatter, fluorescence lifetime, fluorescence polarization, or

a combination of the above parameters. Other detectable responses include, for example, chemiluminescence, phosphorescence, radiation from radioisotopes, magnetic attraction, and electron density.

The term "detectably distinct" as used herein refers to a signal that is distinguishable or separable by a physical property either by observation or by instrumentation. For example, a fluorophore is readily distinguishable either by spectral characteristics or by fluorescence intensity, lifetime, polarization or photo-bleaching rate from another fluorophore in the sample, as well as from additional materials that are optionally present.

The term "directly detectable" as used herein refers to the presence of a material or the signal generated from the material is immediately detectable by observation, instrumentation, or film without requiring chemical modifications or additional substances.

The term "energy transfer" as used herein refers to the process by which the excited state energy of an excited group, e.g. fluorescent reporter dye, is conveyed through space or through bonds to another group, e.g. a quencher moiety or fluorescer, which may attenuate (quench) or otherwise dissipate or transfer the energy to another reporter group or emit the energy at a longer wavelength. Energy transfer typically occurs through fluorescence resonance energy transfer (FRET).

The term "energy transfer pair" as used herein refers to any two moieties that participate in energy transfer. Typically, one of the moieties acts as a fluorescent reporter, i.e. donor, and the other acts as an acceptor, which may be a quenching compound or a compound that absorbs and re-emits energy in the form of a fluorescent signal ("Fluorescence resonance energy transfer." Selvin P. (1995) Methods Enzymol 246:300-334; dos Remedios C. G. (1995) J. Struct. Biol. 115:175-185; "Resonance energy transfer: methods and applications." Wu P. and Brand L. (1994) Anal Biochem 218:1-13). Fluorescence resonance energy transfer (FRET) is a distance-dependent interaction between two moieties in which excitation energy, i.e. light, is transferred from a donor to an acceptor without emission of a photon. The acceptor may be fluorescent and emit the transferred energy at a longer wavelength, or it may be non-fluorescent and serve to diminish the detectable fluorescence of the reporter molecule (quenching). FRET may be either an intermolecular or intramolecular event, and is dependent on the inverse sixth power of the separation of the donor and acceptor, making it useful over distances comparable with the dimensions of biological macromolecules. When an energy transfer pair is part of the present ligand-detection reagent the energy transfer is an intramolecular event. Thus, the spectral properties of the energy transfer pair as a whole

change in some measurable way if the distance between the moieties is altered by some critical amount. Self-quenching probes incorporating fluorescent donor-non-fluorescent acceptor combinations have been developed primarily for detection of proteolysis (Matayoshi, (1990) Science 247:954-958) and nucleic acid hybridization ("Detection of Energy Transfer and Fluorescence Quenching" Morrison, L., in Nonisotopic DNA Probe Techniques, L. Kricka, Ed., Academic Press, San Diego, (1992) pp. 311-352; Tyagi S. (1998) Nat. Biotechnol. 16:49-53; Tyagi S. (1996) Nat. Biotechnol 14:303-308). In most applications, the donor and acceptor dyes are different, in which case FRET can be detected by the appearance of sensitized fluorescence of the acceptor or by quenching of donor fluorescence.

The term "examination zone" as used herein refers to an optical zone of a flow cytometer, or a similar instrument, wherein cells are passed through essentially one at a time in a thin stream whereby the bound immuno-labeled complex is illuminated and the intensity and emission spectra of the fluorophore is detected and recorded. This includes instruments wherein the examination zone moves and the sample is held in place.

The term "fluorophore" as used herein refers to a composition that is inherently fluorescent or demonstrates a change in fluorescence upon binding to a biological compound or metal ion, i.e., fluorogenic. Fluorophores may contain substitutents that alter the solubility, spectral properties or physical properties of the fluorophore. Numerous fluorophores are known to those skilled in the art and include, but are not limited to coumarin, cyanine, benzofuran, a quinoline, a quinazolinone, an indole, a benzazole, a borapolyazaindacene and xanthenes including fluoroscein, rhodamine and rhodol as well as other fluorophores described in RICHARD P. HAUGLAND, MOLECULAR PROBES HANDBOOK OF FLUORESCENT PROBES AND RESEARCH CHEMICALS (9th edition, CD-ROM, September 2002).

The term "immuno-labeled complex" refers to the complex of target-binding antibody that is non-covalently attached to a labeling reagent.

The term "immuno-labeled complex subset" as used herein refers to a discrete set of immuno-labeled complexes that are homogenous and can be distinguished from another subset of immuno-labeled complex by the physical properties of the label, or the ratio of the label to labeling reagent, or the ratio of labeling reagent to target-binding antibody, or the target-binding antibody. Typically an immuno-labeled complex subset is present in a buffer to provide a "target detection solution".

The term "kit" as used herein refers to a packaged set of related components, typically one or more compounds or compositions.

The term "label" as used herein refers to a chemical moiety or protein that retains it's native properties (e.g. spectral properties, conformation and activity) when attached to a labeling reagent and used in the present methods. The label can be directly detectable (fluorophore), indirectly detectable (hapten or enzyme) or act as a quencher for the reporter molecule of the ligand analog. Such labels include, but are not limited to, radiolabels that can be measured with radiation-counting devices; pigments, dyes or other chromogens that can be visually observed or measured with a spectrophotometer; quenching moiety that functions to absorb and not re-emit the energy from the dye moiety of the ligand analog that is within close proximity; spin labels that can be measured with a spin label analyzer; and fluorescent labels (fluorophores), where the output signal is generated by the excitation of a suitable molecular adduct and that can be visualized by excitation with light that is absorbed by the dye or can be measured with standard fluorometers or imaging systems, for example. The label can be a chemiluminescent substance, where the output signal is generated by chemical modification of the signal compound; a metal-containing substance; or an enzyme, where there occurs an enzyme-dependent secondary generation of signal, such as the formation of a colored product from a colorless substrate. The term label can also refer to a "tag" or hapten that can bind selectively to a conjugated molecule such that the conjugated molecule, when added subsequently along with a substrate, is used to generate a detectable signal. For example, one can use biotin as a tag and then use an avidin or streptavidin conjugate of horseradish peroxidate (HRP) to bind to the tag, and then use a colorimetric substrate (e.g., tetramethylbenzidine (TMB)) or a fluorogenic substrate such as Amplex Red reagent (Molecular Probes, Inc.) to detect the presence of HRP. Numerous labels are know by those of skill in the art and include, but are not limited to, particles, fluorophores, haptens, enzymes and their colorimetric, fluorogenic and chemiluminescent substrates and other labels that are described in RICHARD P. HAUGLAND, MOLECULAR PROBES HANDBOOK OF FLUORESCENT PROBES AND RESEARCH PRODUCTS (9th edition, CD-ROM, September 2002), *supra*.

The term "labeling reagent" as used herein refers to a monovalent antibody fragment or a non-antibody monomeric protein provided that the labeling reagent has affinity for a selected region of the target-binding antibody and is covalently attached to a label.

The term "labeling reagent subset" as used herein refers to a discrete set of labeling reagents that are homogenous and can be distinguished from another subset of labeling

reagent either by the physical properties of the label or the ratio of the label to labeling reagent.

The term "labeling solution" as used herein refers to a solution that is used to form an immuno-labeled complex wherein the solution comprises labeling reagents and a buffer.

The term "ligand" as used herein refers to a moiety that contains an antibody binding epitope. The ligand may contain amino acids to form peptides or proteins or the ligand may be essentially free if amino acids. The term "ligand" and "target" as used herein are used interchangeably.

The term "ligand analog" as used herein refers to ligand that has been modified to alter the affinity of the ligand analog for the ligand-binding antibody compared to an appropriate ligand. The affinity modification includes, but is not limited to, the addition of a dye moiety, addition of alkyl groups to the binding epitope, change of amino acid sequence of the epitope or spacing of the dye moiety from the epitope. Thus, the ligand analog has the same spatial and polar organization as the ligand to define one or more determinant or epitopic sites capable of competing with the ligand for the binding sites of a receptor, and differs from the ligand in the absence or presence of an atom or functional group at the site of binding to another molecule or in having a linking group which has been introduced in place of one or more atoms originally present in the ligand.

The term "ligand-binding antibody" as used herein refers to an antibody that has affinity for a discrete epitope, antigen or ligand that can be used with the methods of the present invention. Typically the discrete epitope is the target but the epitope can be a marker for the target such as CD3 on T cells. Ligand-binding antibody can be used interchangeably with the term "primary antibody" when describing methods that use an antibody that binds directly to the antigen as opposed to a "secondary antibody" that binds to a region of the primary antibody. As used herein the term "ligand-binding antibody" is used interchangeably with "target-binding antibody".

The term "ligand-detection reagent" as used herein refers to an immuno-complex that is used to determine the presence of a target ligand in a sample. The complex comprises a ligand-binding antibody, a ligand analog and a labeling reagent wherein the covalently bonded label is a fluorophore or a quenching moiety. In this instance, the ligand analog is

displaced by the target ligand resulting in a change in signal intensity or a shift in color change of the detectable signal whereby the presence of a target ligand is determined.

The term "matrix" as used herein refers to a solid or semi-solid surface that a biological molecule can be attached to, such as a sample of the present invention or a capture reagent. Examples include, but are not limited to, agarose, polyacrylamide gel, polymers, microspheres, glass surface, plastic surface, membrane, margnetic surface, and an array.

The term "monovalent antibody fragment" as used herein refers to an antibody fragment that has only one antigen-binding site. Examples of monovalent antibody fragments include, but are not limited to, Fab fragments (no hinge region), Fab' fragments (monovalent fragments that contain a heavy chain hinge region), and single-chain fragment variable (ScFv) proteins.

The term "non-antibody monomeric protein" as used herein refers to a protein that binds selectively and non-covalently to a member of the Ig superfamily of proteins, including but not limited to proteins A, G, and L, hybrids thereof (A/G), recombinant versions and cloned versions thereof, fusions of these proteins with detectable protein labels, and lectins but the protein itself is not an antibody or an antibody fragment.

The terms "protein" and "polypeptide" are used herein in a generic sense to include polymers of amino acid residues of any length. The term "peptide" is used herein to refer to polypeptides having less than 100 amino acid residues, typically less than 10 amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residues are an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers.

The term "purified" as used herein refers to a preparation of a target-binding antibody that is essentially free from contaminating proteins that normally would be present in association with the antibody, e.g., in a cellular mixture or milieu in which the protein or complex is found endogenously such as serum proteins or hybridoma supernatant.

The term "quenching moiety" or "quencher" as used herein refers to a compound that is capable of absorbing energy from an energy donor that is not re-emitted (non-fluorescent) or re-emitted at a detectably different wavelength from the energy emitted by the donor molecule. In this respect, quenchers may be essentially non-fluorescent or fluorescent. Numerous quenching moieties are well known in the art including xanthene and cyanine compounds and other compounds disclosed in RICHARD P. HAUGLAND, MOLECULAR

PROBES HANDBOOK OF FLUORESCENT PROBES AND RESEARCH PRODUCTS (9th edition, CD-ROM, September 2002), *supra*.

The term "reporter molecule" as used herein refers to any luminescent molecule that is capable of functioning as a member of an energy transfer pair wherein the reporter molecule retains it's native properties (e.g. spectral properties, conformation and activity) when attached to a ligand analog and used in the present methods. Typically, luminescent molecules, as used herein include dyes, fluorescent proteins, phosphorescent dyes, chromophores and chemiluminescent compounds that are capable of producing a detectable signal upon appropriate activation. The term "dye" refers to a compound that emits light to produce an observable detectable signal. "Dye" includes fluorescent and nonfluorescent compounds that include without limitations pigments, fluorophores, chemiluminescent compounds, luminescent compounds and chromophores. The term "chromophore" as used herein refers to a label that emits light in the visible spectra that can be observed without the aid of instrumentation. The term "fluorophore" as used herein refers to a composition that is inherently fluorescent or demonstrates a change in fluorescence upon binding to a biological compound, i.e. can be fluorogenic or the intensity can be diminished by quenching. Fluorophores may contain substitutents that alter the solubility, spectral properties or physical properties of the fluorophore. Numerous fluorophores are known to those skilled in the art and include, but are not limited to coumarin, cyanine, benzofuran, a quinoline, a quinazolinone, an indole, a benzazole, a borapolyazaindacene and xanthenes including fluoroscein, rhodamine and rhodol as well as other fluorophores described in RICHARD P. HAUGLAND, MOLECULAR PROBES HANDBOOK OF FLUORESCENT PROBES AND RESEARCH CHEMICALS (9th edition, CD-ROM, September 2002).

The term "sample" as used herein refers to any material that may contain a target, as defined below. Typically, the sample comprises a population of cells, cellular extract, subcellular components, tissue culture, a bodily fluid, and tissue. The sample may be in an aqueous solution, a viable cell culture or immobilized on a solid or semi solid surface such as a gel, a membrane, a glass surface, a microparticle or on a microarray.

The term "target" as used herein refers to any entity that a target-binding antibody has affinity for such as an epitope or antigen. This target includes not only the discrete epitope that the target-binding antibody has affinity for but also includes any subsequently bound molecules or structures. In this way an epitope serves as a marker for the intended target.

For example, a cell is a target wherein the target-binding antibody binds a cell surface protein such as CD3 on a T cell wherein the target marker is CD3 and the target is the T cell.

The term "target-binding antibody" as used herein refers to an antibody that has affinity for a discrete epitope or antigen that can be used with the methods of the present invention. Typically the discrete epitope is the target but the epitope can be a marker for the target such as CD3 on T cells. The term can be used interchangeably with the term "primary antibody" when describing methods that use an antibody that binds directly to the antigen as opposed to a "secondary antibody" that binds to a region of the primary antibody.

The term "ternary complex" as used herein refers to a composition that simultaneously comprises a ligand-binding antibody, a ligand analog of the present invention and labeling reagent wherein the ligand analog is non-covalently bound in the binding groove of the ligand-binding antibody and the labeling reagent is non-covalently bonded to a region, e.g. Fc, of the ligand-binding antibody.

II. COMPOSITIONS AND METHODS OF USE

In accordance with the present invention, labeling reagents, methods for labeling target-binging antibodies and methods for using the labeled antibodies to detect a target in a sample including a target ligand in a competitive immunoassay are provided. The labeling reagents comprise monovalent antibody fragments or non-antibody monomeric proteins that are covalently attached to a label of the present invention. The label covalently attached to a labeling reagent is directly detectable such as a fluorophore, a quenching moiety or functions as an indirect label that requires an additional component such as a colorimetric enzyme substrate or an enzyme conjugate. The labeling reagents have affinity for a specific region of the target-binding antibody. The target-binding antibodies are defined as any antibody known to one skilled in the art that has an affinity for a target in a sample. The target-binding antibodies are labeled with the labeling reagent in a labeling method to form immuno-labeled complexes and then added to a sample to detect a target.

The labeling reagent and the methods of the present invention provide for detection of one or multiple targets in a sample. Multiple targets are detected when either pooled subsets of immuno-labeled complexes or a panel of subsets that are sequentially added to a sample. The subset of immuno-labeled complexes begins with labeling reagent subsets wherein a

labeling reagent subset is distinguished by the ratio of label to labeling reagent or by the physical characteristics of the label. The discrete labeling reagents subsets are added to the target-binding antibodies wherein the affinity of the antibody and ratio of labeling reagent to target-binding antibody determines the subsets of immuno-labeled complexes. This results in an infinite number of immuno-labeled complex subsets that are distinguished by i) the target-binding antibody, or ii) a ratio of label to labeling reagent, or iii) a ratio of labeling reagent to the target-binding antibody or iv) by a physical property of the label. These subsets can be used individually in a method of the present invention to detect a single or multiple targets in a sample or pooled and used to simultaneously detect multiple targets in a sample. These pooled subsets allow for not only detection but also identification and quantitation of the targets.

In one aspect of the invention, reagent and methods are provided for the detection of a target ligand employing a competitive immunoassay wherein a ligand analog is displaced by a target ligand resulting in a change in detectable signal that indicates the presence of target ligand. Thus, the present invention provides ligand-detection reagents, ligand analogs and methods of employing the reagents for the detection of a target ligand. The ligand-detection reagents comprise a ligand-binding antibody, a ligand analog that is covalently attached to a reporter molecule and a labeling reagent that is covalently attached to a label. The ligand-detection reagent is a complex wherein the ligand analog is non-covalently bound by the binding groove of the antibody and the labeling reagent is non-covalently bound to a region of the antibody. The methods employ the ligand-detection reagents wherein in one aspect of the invention energy transfer is utilized in a competitive immunoassay format to determine the presence of a ligand in a sample.

A. Ligand Analog

The ligand analog comprises at least one epitope site for a desired ligand-binding antibody, a reporter molecule and a linker. Thus, the ligand analog may be monovalent or polyvalent. Typically the ligand analog is monovalent or divalent. In one aspect of the invention the monovalent ligand analog is quenched when bound by the ligand-binding antibody. Thus, monovalent ligand analogs are preferred for applications wherein it is desired that the ligand analog be quenched when bound by the ligand-binding antibody. In another aspect, the divalent ligand analog is fluorogenic wherein the ligand analog is essentially non-fluorescent when unbound from the ligand-binding antibody but when bound by the antibody in such a way that the each binding groove is bound to the bivalent ligand analog and the reporter molecule is held in between the two Fab fragments of the antibody the fluorogenic ligand

analog becomes fluorescent, *See* Example 32 and Compounds **4** and **5**. Thus, in this aspect a divalent ligand analog is preferred for applications wherein a fluorogenic ligand analog is employed.

The ligand analog typically has an altered affinity for the ligand-binding antibody compared to the target ligand. The altered affinity may be greater or less than the target ligand, typically the affinity is less or equal to the affinity of the target ligand. The affinity of the ligand analog is determined empirically along with the selection of the ligand-binding antibody, and optionally the labeling protein to optimize the displacement of the ligand analog by the target ligand in each assay system. The altered affinity of the ligand analog can be accomplished by a number of modifications to the target ligand to make a ligand analog or alternatively a synthetic chemical strategy can be employed to design and synthesize a ligand analog with the appropriate affinity, fluorescence response, and ability to be quenched. Modification to a ligand to form a ligand analog can include a change of a single, or multiple, amino acids, either in the epitope or the surrounding sequence, a change in the post-translational modification of a protein or peptide such as the addition or removal of a sugar group or phosphate, the addition of a linker or simply by the addition of a reporter molecule. Alternatively, the epitope can be synthesized with an appropriate linker and reporter molecule, such as was done for the phosphotyramide, phosphotyrosinamide, phosphoethanoamine and phosphoserine ligand analogs, *See* Examples 22-29.

Synthesis of the epitope, linker and reporter group provide for the most flexibility for designing a ligand analog with the appropriate affinity for the ligand-binding antibody and spectral properties of the reporter group. However, this method is typically not preferred wherein a ligand-binding antibody was raised against a ligand that requires a conformational epitope. In this instance, the sequence, spacing, and folding or conformation of the antigen is necessary for adequate binding by the ligand-binding antibody. Thus, for these ligand analogs, the reporter molecule is typically conjugated to the target ligand to form the analog resulting in linker that is typically less than 10 atoms in length.

The ligand analog for the most part will be haptenic, rather than antigenic, and generally be less than about 10,000 molecular weight, more usually less than about 6,000 molecular weight, and frequently in the range of about 125 to 1,000 molecular weight, excluding the linking group employed for linking to the reporter molecule.

Regardless of the method employed to derive a ligand analog, the reporter molecule is typically conjugated to the ligand analog. Thus, the reporter molecule and the ligand analog

each need to contain an appropriate reactive or functional group that result in a covalent bond. The reactive group and functional group are typically an electrophile and a nucleophile that can generate a covalent linkage. Alternatively, the reactive group is a photoactivatable group, and becomes chemically reactive only after illumination with light of an appropriate wavelength. Typically, the conjugation reaction between the reactive group of the reporter molecule and the reactive group of the ligand analog results in one or more atoms of the reactive group to be incorporated into a new linkage attaching the reporter molecule to the ligand analog. Selected examples of functional groups and linkages are shown in Table 1, where the reaction of an electrophilic group and a nucleophilic group yields a covalent linkage.

Table 1: Examples of some routes to useful covalent linkages

Electrophilic Group	Nucleophilic Group	Resulting Covalent Linkage
activated esters*	amines/anilines	carboxamides
acrylamides	thiols	thioethers
acyl azides**	amines/anilines	carboxamides
acyl halides	amines/anilines	carboxamides
acyl halides	alcohols/phenols	esters
acyl nitriles	alcohols/phenols	esters
acyl nitriles	amines/anilines	carboxamides
aldehydes	amines/anilines	imines
aldehydes or ketones	hydrazines	hydrazones
aldehydes or ketones	hydroxylamines	oximes
alkyl halides	amines/anilines	alkyl amines
alkyl halides	carboxylic acids	esters
alkyl halides	thiols	thioethers
alkyl halides	alcohols/phenols	ethers
alkyl sulfonates	thiols	thioethers
alkyl sulfonates	carboxylic acids	esters
alkyl sulfonates	alcohols/phenols	ethers
anhydrides	alcohols/phenols	esters
anhydrides	amines/anilines	carboxamides
aryl halides	thiols	thiophenols
aryl halides	amines	aryl amines
aziridines	thiols	thioethers

boronates	glycols	boronate esters
carbodiimides	carboxylic acids	N-acylureas or anhydrides
diazoalkanes	carboxylic acids	esters
epoxides	thiols	thioethers
haloacetamides	thiols	thioethers
haloplatinate	amino	platinum complex
haloplatinate	heterocycle	platinum complex
haloplatinate	thiol	platinum complex
halotriazines	amines/anilines	aminotriazines
halotriazines	alcohols/phenols	triazinyl ethers
halotriazines	thiols	triazinyl thioethers
imido esters	amines/anilines	amidines
isocyanates	amines/anilines	ureas
isocyanates	alcohols/phenols	urethanes
isothiocyanates	amines/anilines	thioureas
maleimides	thiols	thioethers
phosphoramidites	alcohols	phosphite esters
silyl halides	alcohols	silyl ethers
sulfonate esters	amines/anilines	alkyl amines
sulfonate esters	thiols	thioethers
sulfonate esters	carboxylic acids	esters
sulfonate esters	alcohols	ethers
sulfonyl halides	amines/anilines	sulfonamides
sulfonyl halides	phenols/alcohols	sulfonate esters

* Activated esters, as understood in the art, generally have the formula -COΩ, where Ω is a good leaving group (e.g., succinimidyloxy ($-OC_4H_4O_2$) sulfosuccinimidyloxy ($-OC_4H_3O_2$-SO_3H), -1-oxybenzotriazolyl ($-OC_6H_4N_3$); or an aryloxy group or aryloxy substituted one or more times by electron withdrawing substituents such as nitro, fluoro, chloro, cyano, or trifluoromethyl, or combinations thereof, used to form activated aryl esters; or a carboxylic acid activated by a carbodiimide to form an anhydride or mixed anhydride $-OCOR^a$ or $-OCNR^aNHR^b$, where R^a and R^b, which may be the same or different, are C_1-C_6 alkyl, C_1-C_6 perfluoroalkyl, or C_1-C_6 alkoxy; or cyclohexyl, 3-dimethylaminopropyl, or N-morpholinoethyl).

** Acyl azides can also rearrange to isocyanates

Choice of the reactive group used to attach the reporter molecule to the ligand analog typically depends on the reactive or functional group on the ligand analog and the type or length of covalent linkage desired. The types of functional groups typically present on biomolecules include, but are not limited to, amines, amides, thiols, alcohols, phenols, aldehydes, ketones, phosphates, imidazoles, hydrazines, hydroxylamines, disubstituted amines, halides, epoxides, silyl halides, carboxylate esters, sulfonate esters, purines, pyrimidines, carboxylic acids, olefinic bonds, or a combination of these groups. A single type of reactive site may be available on the substance (typical for polysaccharides or silica), or a variety of sites may occur (e.g., amines, thiols, alcohols, phenols), as is typical for proteins.

A linker may be synthesized on the ligand analog or on the reporter molecule wherein after conjugation the linker is incorporated into the ligand analog. The linker typically incorporates 1-30 nonhydrogen atoms selected from the group consisting of C, N, O, S and P. The linker is optionally a substituted alkyl, amine or a substituted cycloalkyl. Alternately, the reporter group may be directly attached (where linker is a single bond) to the ligand analog or the alkyl may contain a benzene ring. When the linker is not a single covalent bond, the linker may be any combination of stable chemical bonds, optionally including, single, double, triple or aromatic carbon-carbon bonds, as well as carbon-nitrogen bonds, nitrogen-nitrogen bonds, carbon-oxygen bonds, sulfur-sulfur bonds, carbon-sulfur bonds, phosphorus-oxygen bonds, phosphorus-nitrogen bonds, and nitrogen-platinum bonds. Typically the linker incorporates less than 20 nonhydrogen atoms and are composed of any combination of ether, thioether, thiourea, amine, ester, carboxamide, sulfonamide, hydrazide bonds and aromatic or heteroaromatic bonds. Most preferred are linkers that contain less than 10 non-hydrogen atoms. Typically the linker is a combination of single carbon-carbon bonds and carboxamide, sulfonamide or thioether bonds. The bonds of the linker typically result in the following moieties that can be found in the linker: ether, thioether, carboxamide, thiourea, sulfonamide, urea, urethane, hydrazine, alkyl, aryl, heteroaryl, alkoky, cycloalkyl and amine moieties.

Any combination of linkers may be used to attach the reporter molecule to the ligand analog. For monovalent ligand analogs the analog typically contains one linker and for divalent ligand analogs the analogs typically incorporate two linkers, which may be the same or different. The linker may also be substituted to alter the physical properties of the ligand analog, such as binding affinity for the ligand-binding antibody and spectral properties of the fluorophore.

We have unexpectedly discovered that the site of attachment of the linker on the reporter group alters the ability of the reporter molecule to be quenched when bound to the ligand-binding antibody and the binding affinity of the analog for the ligand-binding antibody. This is particularly true when the reporter molecule is a xanthene dye. By way of example Compound **8** and **9** are isomers but demonstrate different binding affinity for the same antibody and quenching by the same antibody, *See,* Example 43 and Figure 19. In this instance, the 6-isomer (linker is attached to the 6 position of the pendent phenyl ring; Compound **9**) demonstrates increased binding affinity and increased quenching compared to the 5-isomer of the same ligand analog, Compound **8**. Therefore, the position of attachment of the linker on the reporter molecule is important for defining binding affinity of the ligand analog for the ligand-binding antibody and for the ability of the reporter molecule to be masked or quenched when bound by the ligand-binding antibody. This quenching property is particularly relevant when a monovalent ligand analog is employed.

The length of the linker is another important aspect for optimizing the amount of quenching conferred on the reporter molecule. We have found that a shorter linker results in an increased quenching of the reporter molecule by the ligand-binding antibody. Without wishing to be bound by a theory, it appears that quenching is increased when the reporter molecule is "pulled" into the binding groove of the antibody, which is facilitated by a short linker; a short linker preferably containing 10 or less non-hydrogen atoms. In addition, the linker can be substituted by substitutents that alter the physical properties of the ligand analog, such as binding affinity and spectral properties of the reporter molecule. We have unexpectedly found that substituting the linker to form a phosphotyrosinamide instead of a phosphotyramide ligand analog alters the binding affinity and the ability of the reporter molecule to be quenched when bound by the ligand-binding antibody. *See*, Compounds **34-38** and **41-42**and Example 44.

Therefore, the linker of the ligand analog is important for attaching the reporter molecule to the ligand analog, for altering the binding affinity of the analog and for altering the spectral properties of the reporter group. The lengths of the linker, site of attachment on the reporter group and linker substituents all are parameters that can be altered to maximize the binding affinity of the analog for the antibody and the ability of the reporter molecule to be quenched when bound by the ligand-binding antibody.

The reporter molecules of the present invention include any detectable label known by one skilled in the art that can be covalently attached to the ligand analog of the present invention. When part of the ligand analog, the reporter molecule is typically capable of transferring

energy to another moiety to be absorbed and optionally re-emitted at a longer wavelength. Alternatively, the reporter molecule is fluorogenic such that when the ligand analog is bound to the ligand-binding antibody the reporter group is fluorescent but when unbound is essentially non-fluorescent. Reporter molecules include, without limitation, a chromophore, a fluorophore, a fluorescent protein, and a phosphorescent dye. Typically, substituents on the fluorophore or ligand analog alter the spectral properties to form a fluorogenic ligand analog. Preferred reporter molecules include fluorophores and fluorescent proteins.

A fluorophore of the present invention is any chemical moiety that exhibits an absorption maximum beyond 280 nm, and when covalently attached to a ligand analog retains its spectral properties. Fluorophores of the present invention include, without limitation; a pyrene (including any of the corresponding derivative compounds disclosed in US Patent 5,132,432), an anthracene, a naphthalene, an acridine, a stilbene, an indole or benzindole, an oxazole or benzoxazole, a thiazole or benzothiazole, a 4-amino-7-nitrobenz-2-oxa-1, 3-diazole (NBD), a cyanine (including any corresponding compounds in US Serial Nos. 09/968,401 and 09/969,853), a carbocyanine (including any corresponding compounds in US Serial Nos. 09/557,275; 09/969,853 and 09/968,401; U.S.; Patents Nos. 4,981,977; 5,268,486; 5,569,587; 5,569,766; 5,486,616; 5,627,027; 5,808,044; 5,877,310; 6,002,003; 6,004,536; 6,008,373; 6,043,025; 6,127,134; 6,130,094; 6,133,445; and publications WO 02/26891, WO 97/40104, WO 99/51702, WO 01/21624; EP 1 065 250 A1), a carbostyryl, a porphyrin, a salicylate, an anthranilate, an azulene, a perylene, a pyridine, a quinoline, a borapolyazaindacene (including any corresponding compounds disclosed in US Patent Nos. 4,774,339; 5,187,288; 5,248,782; 5,274,113; and 5,433,896), a xanthene (including any corresponding compounds disclosed in U.S. Patent No. 6,162,931; 6,130,101; 6,229,055; 6,339,392; 5,451,343 and US serial No. 09/922,333), an oxazine (including any corresponding compounds disclosed in US Patent No. 4,714,763) or a benzoxazine, a carbazine (including any corresponding compounds disclosed in US Patent No. 4,810,636), a phenalenone, a coumarin (including an corresponding compounds disclosed in US Patent Nos. 5,696,157; 5,459,276; 5,501,980 and 5,830,912), a benzofuran (including an corresponding compounds disclosed in US Patent Nos. 4,603,209 and 4,849,362) and benzphenalenone (including any corresponding compounds disclosed in US Patent No. 4,812,409) and derivatives thereof. As used herein, oxazines include resorufins (including any corresponding compounds disclosed in 5,242,805), aminooxazinones, diaminooxazines, and their benzo-substituted analogs.

When the fluorophore is a xanthene, the fluorophore is optionally a fluorescein, a rhodol (including any corresponding compounds disclosed in US Patent Nos. 5,227,487 and

5,442,045), or a rhodamine (including any corresponding compounds in US Patent Nos. 5,798,276; 5,846,737; US serial no. 09/129,015). As used herein, fluorescein includes benzo- or dibenzofluoresceins, seminaphthofluoresceins, or naphthofluoresceins. Similarly, as used herein rhodol includes seminaphthorhodafluors (including any corresponding compounds disclosed in U.S. Patent No. 4,945,171). Alternatively, the fluorophore is a xanthene that is bound via a linkage that is a single covalent bond at the 9-position of the xanthene. Preferred xanthenes include derivatives of 3*H*-xanthen-6-ol-3-one attached at the 9-position, derivatives of 6-amino-3*H*-xanthen-3-one attached at the 9-position, or derivatives of 6-amino-3*H*-xanthen-3-imine attached at the 9-position.

Preferred fluorophores of the invention include xanthene (rhodol, rhodamine, fluorescein and derivatives thereof) coumarin, cyanine, pyrene, oxazine and borapolyazaindacene. Most preferred are fluorinated xanthenes, fluorinated coumarins and cyanines. When conjugated to a ligand analog it is preferred that the fluorophore not be substituted by a polar group such as SO_3^- due to poor binding affinity conferred to the ligand analog for the ligand-binding antibody. The choice of the fluorophore attached to the ligand analog will determine the absorption and fluorescence emission properties of the ligand analog, the ligand-detection reagent and ultimately the assay solution in the presence of a ligand. Physical properties of a fluorophore label include spectral characteristics (absorption, emission and stokes shift), fluorescence intensity, lifetime, polarization and photo-bleaching rate all of which can be used to distinguish one fluorophore from another.

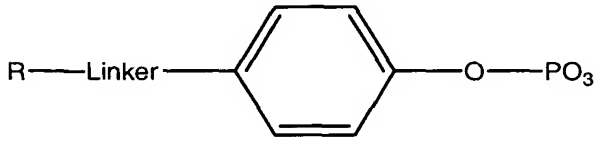
Typically the fluorophore contains one or more aromatic or heteroaromatic rings, that are optionally substituted one or more times by a variety of substituents, including without limitation, halogen, nitro, cyano, alkyl, perfluoroalkyl, alkoxy, alkenyl, alkynyl, cycloalkyl, arylalkyl, acyl, aryl or heteroaryl ring system, benzo, or other substituents typically present on fluorophores known in the art.

In one aspect of the invention, the fluorophore has an absorption maximum beyond 480 nm. In a particularly useful embodiment, the fluorophore absorbs at or near 488 nm to 514 nm (particularly suitable for excitation by the output of the argon-ion laser excitation source) or near 546 nm (particularly suitable for excitation by a mercury arc lamp).

Many of fluorophores can also function as chromophores and thus the described fluorophores are also preferred chromophores of the present invention.

In one aspect of the invention the ligand analogs are phospho-tyrosine, -threonine or -serine ligand analogs that are employed for the detection of phosphorylated biomolecules including proteins and peptides or for the detection of kinase or phosphatase enzyme activity. Preferred phospho- ligand analogs typically comprise a phosphotyramide moiety or a phosphoethanolamide moiety and include phosphotyramide, phosphotyrosinamide and phosphoethanolamide ligand analogs.

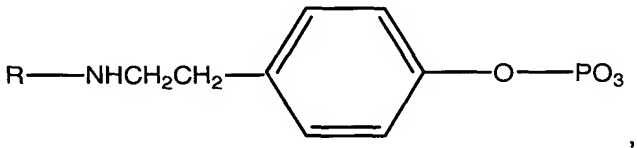
Typically a ligand analog comprising a phosphophenol moiety has the following formula



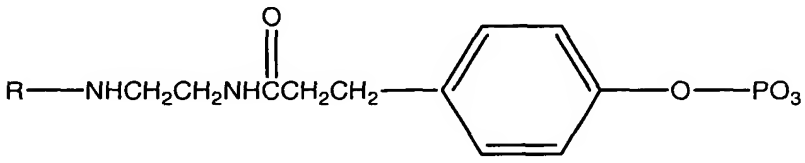
Formula I

wherein R is a reporter molecule and linker is a single covalent bond or comprises 1-20 non-hydrogen atoms to covalently attach the reporter molecule to the phosphophenol moiety. Preferred reporter molecules include borapolyazaindacene, coumarin, xanthene, cyanine, fluorescent protein and phosphorescent dye. Most preferred reporter molecules are xanthene, borapolyazaindacene and coumarin, typically these reporter molecules are not substituted by polar groups. The linker typically contains alkyl and amine groups.

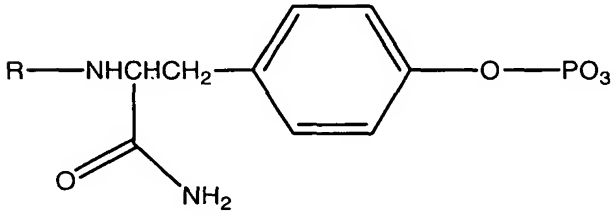
In one embodiment, ligand analogs comprising a phosphotyramide moiety are selected from the group consisting of:



,Formula II



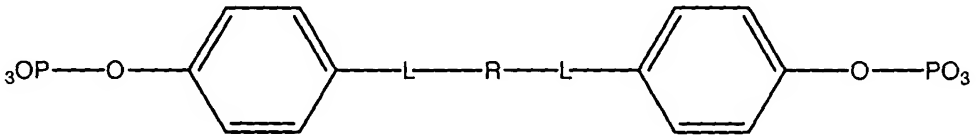
, Formula III and



Formula IV.

Exemplified Compounds according to Formula II include Compounds **2, 7-17, 20-21 and 39**, exemplified Compounds according to Formula III include Compounds **22-33** and **40**, and exemplified compounds according to Formula IV include compounds **34-38** and **41-42**. Formula IV, as used herein is typically referred to as a phosphotyrosinamide ligand analog.

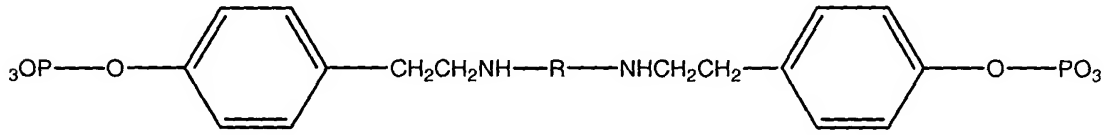
Alternatively, the phosphophenol moiety forms part of a fluorogenic ligand analog according to formula:



Formula V

wherein R is a reporter molecule and L is a linker that is a single covalent bond or comprises 1-20 non-hydrogen atoms to covalently attached said reporter molecule to phosphophenol moiety. Again, preferred reporter molecules are selected from the group consisting of borapolyazaindacene, coumarin, xanthene, cyanine, fluorescent protein and phosphorescent dye. Most preferred is a borapolyazaindacene reporter molecule, See Compounds **4** and **5**. The linker typically comprises alkyl and amino groups.

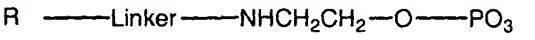
Thus, in a preferred embodiment a fluorogenic ligand analog comprising a phosphotyramide moiety is according to formula:



Formula VI.

The fluorogenic ligand analogs are not limited to phosphotyramide moieties; it is appreciated that any epitope that has affinity for a ligand-binding antibody can replace the phosphotyramide moiety of formula VI to form a fluorogenic ligand analog. Preferred epitopes are single amino acids or portions thereof including theronine and serine.

In another aspect of the invention, the ligand analog has affinity for a phosphothreonine or phosphoserine ligand-binding antibody. In this instance, the ligand analogs are typically phosphoethanolamines according to formula



Formula VII

wherein R is a reporter molecule and linker is a single covalent bond or comprises 1-20 non-hydrogen atoms to covalently attached said reporter molecule to phosphoethanolamine moiety. Typically the linker is a single covalent bond, See Compound **2**.

In addition, it is also contemplated that serine and threonine residues conjugated to a reporter molecule also form part of the invention, See Compound **43** Example 29.

Theses non-fluorogenic ligand analogs that comprise phosphoethanolamine, serine, threonine and phosphophenol (phosphotyramide) moieties are all capable of being quenched when bound by a ligand-binding antibody. Preferred ligand analogs that demonstrate a high degree of quenching typically comprise a xanthene reporter molecule wherein the linker is attached at the 6-position of the pendent phenyl ring including Compounds **7, 9, 12, 15, 19, 23, 25, 27, 33, 34** and **38**. Most preferred ligand analogs for their ability to be quenched when bound by a phosphotyrosine ligand-binding antibody are Compounds **9**, **15**, **23** and **34**.

B. Labeling Reagents

In addition to forming immune-complexes for the detection of a target in a sample, the labeling reagents also form part the ligand-detection reagent wherein the labeling reagent comprises a monovalent antibody fragment or a non-antibody protein and a covalently bound label. Labels that are conjugated to a ligand analog are selected from the group consisting of a chromophore, a fluorophore, a quenching moiety, a fluorescent protein and a phosphorescent dye. Typically the label is a fluorophore or a quenching moiety that is capable of absorbing energy from the reporter molecule of the ligand analog when bound by the ligand-binding antibody. The absorbed energy is either quenched (not re-emitted) or re-emitted at a longer wavelength resulting is a color shift of the detectable signal.

The labeling reagents of the present invention are monovalent antibody fragments or non-antibody monomeric proteins that have affinity for a region of a target-binding antibody. The regions of the target-binding antibody that can be bound by a labeling reagent include the Fc region, Fab region, the kappa or lambda light chain region or a heavy chain region. When the labeling reagent is derived from an antibody the monovalent fragment can be, anti-Fc, anti-Fab, an anti–Fc isotype, anti–kappa light chain, anti–lambda light chain, or a single-chain fragment variable protein. Labeling reagents that are a non-antibody peptide or

protein, are for example but not limited to, soluble Fc receptor, protein G, protein A, protein L, lectins, or a fragment thereof. The labeling reagents typically have affinity for the Fc region of the target-binding antibody but any region, except the binding domain, may be used as a binding site for the labeling reagent. The Fc region is preferable because it is the farthest from the binding domain of the target-binding antibody and is unlikely to cause steric hinderance, when bound by a labeling reagent, of the binding domain for the target.

Antibody is a term of the art denoting the soluble substance or molecule secreted or produced by an animal in response to an antigen, and which has the particular property of combining specifically with the antigen that induced its formation. Antibodies themselves also serve are antigens or immunogens because they are glycoproteins and therefore are used to generate anti-species antibodies. Antibodies, also known as immunoglobulins, are classified into five distinct classes--IgG, IgA, IgM, IgD, and IgE. The basic IgG immunoglobulin structure consists of two identical light polypeptide chains and two identical heavy polypeptide chains (linked together by disulfide bonds). When IgG is treated with the enzyme papain, a monovalent antigen-binding fragment can be isolated, referred herein to as a Fab fragment. When IgG is treated with pepsin (another proteolytic enzyme), a larger fragment is produced, $F(ab')_2$. This fragment can be split in half by treating with a mild reducing buffer that results in the monovalent Fab' fragment. The Fab' fragment is slightly larger than the Fab and contains one or more free sulfhydryls from the hinge region (which are not found in the smaller Fab fragment). The term "antibody fragment" is used herein to define both the Fab' and Fab portions of the antibody. It is well known in the art to treat antibody molecules with pepsin and papain in order to produce antibody fragments (Gorevic *et al.*, Methods of Enzyol., 116:3 (1985)).

The monovalent Fab fragments of the present invention are produced from either murine monoclonal antibodies or polyclonal antibodies generated in a variety of animals that have been immunized with a foreign antibody or fragment thereof, US Patent No. 4,196,265 discloses a method of producing monoclonal antibodies. Typically, labeling reagents are derived from a polyclonal antibody that has been produced in a rabbit or goat but any animal known to one skilled in the art to produce polyclonal antibodies can be used to generate anti-species antibodies. However, monoclonal antibodies are equal, and in some cases, preferred over polyclonal antibodies provided that the target-binding antibody is compatible with the monoclonal antibodies that are typically produced from murine hybridoma cell lines using methods well known to one skilled in the art. Example 1 describes production of polyclonal antibodies raised in animals immunized with the Fc region of a foreign antibody. It is a preferred embodiment of the present invention that the labeling reagents be generated

against only the Fc region of a foreign antibody. Essentially, the animal is immunized with only the Fc region fragment of a foreign antibody, such as murine. The polyclonal antibodies are collected from subsequent bleeds, digested with an enzyme, pepsin or papain, to produce monovalent fragments. The fragments are then affinity purified on a column comprising whole immunoglobulin protein that the animal was immunized against or just the Fc fragments. As described in detail below, the labeling reagents are also covalently labeled with fluorophore labels when bound to the affinity column to eliminate incorporating label into the binding domain of the monovalent fragment. One of skill in the art will appreciate that this method can be used to generate monovalent fragments against any region of a target-binding protein and that selected peptide fragments of the target-binding antibody could also be used to generate fragments.

Alternatively, a non-antibody protein or peptide such as protein G, or other suitable proteins, can be used alone or coupled with albumin wherein albumin is attached with a label of the present invention. Preferred albumins of the invention include human and bovine serum albumins or ovalbumin. Protein A, G and L are defined to include those proteins know to one skilled in the art or derivatives thereof that comprise at least one binding domain for IgG, i.e. proteins that have affinity for IgG. These proteins can be modified but do not need to be and are labeled in the same manner as the monovalent Fab fragments of the invention.

The labels of the present invention include any directly or indirectly detectable label known by one skilled in the art that can be covalently attached to the labeling reagent of the present invention. Labels include, without limitation, a chromophore, a fluorophore, a fluorescent protein, a phosphorescent dye, a tandem dye, a particle, a hapten, an enzyme and a radioisotope. Preferred labels include fluorophores, fluorescent proteins, haptens, and enzymes.

For labels (labeling reagent) that are to be used with a ligand-binding antibody and a ligand analog, the labels, by definition, are capable of absorbing energy from the reporter molecule when the ligand analog is bound by the ligand-binding antibody. The labels included a chromophore, a fluorophore, a quenching moiety, a fluorescent protein and a phosphorescent dye. Typically, these labels include fluorophores and quenching moieties, which include both fluorescent and essentially non-fluorescent compounds. The fluorophores (and chromophores) can be any of the compounds disclosed above for use as a reporter molecule including compounds substituted with polar groups.

Numerous quenching compounds are known to one of skill in the art including, but not limited to, compounds disclosed in US Patent No. 6,541,618 and US Serial No. 09/942,342 and cyanine compounds disclosed in US Patent Nos. 6,348,596; 6,080,868 and US Serial No. 60/491,783, xanthene compounds US Patent No. 6,399,392.

In addition to fluorophores, enzymes also find use as labels for the labeling reagents. Enzymes are desirable labels because amplification of the detectable signal can be obtained resulting in increased assay sensitivity. The enzyme itself does not produce a detectable response but functions to break down a substrate when it is contacted by an appropriate substrate such that the converted substrate produces a fluorescent, colorimetric or luminescent signal. Enzymes amplify the detectable signal because one enzyme on a labeling reagent can result in multiple substrates being converted to a detectable signal. This is advantageous where there is a low quantity of target present in the sample or a fluorophore does not exist that will give comparable or stronger signal than the enzyme. However, fluorophores are most preferred because they do not require additional assay steps and thus reduce the overall time required to complete an assay. The enzyme substrate is selected to yield the preferred measurable product, e.g. colorimetric, fluorescent or chemiluminescence. Such substrates are extensively used in the art, many of which are described in the MOLECULAR PROBES HANDBOOK, *supra*.

A preferred colorimetric or fluorogenic substrate and enzyme combination uses oxidoreductases such as horseradish peroxidase and a substrate such as 3,3'-diaminobenzidine (DAB) and 3-amino-9-ethylcarbazole (AEC), which yield a distinguishing color (brown and red, respectively). Other colorimetric oxidoreductase substrates that yield detectable products include, but are not limited to: 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), *o*-phenylenediamine (OPD), 3,3',5,5'-tetramethylbenzidine (TMB), *o*-dianisidine, 5-aminosalicylic acid, 4-chloro-1-naphthol. Fluorogenic substrates include, but are not limited to, homovanillic acid or 4-hydroxy-3-methoxyphenylacetic acid, reduced phenoxazines and reduced benzothiazines, including Amplex® Red reagent and its variants (U.S. Pat. No. 4,384,042) and reduced dihydroxanthenes, including dihydrofluoresceins (U.S. Pat. No. 6,162,931) and dihydrorhodamines including dihydrorhodamine 123. Peroxidase substrates that are tyramides (U.S. Pat. Nos. 5,196,306; 5,583,001 and 5,731,158) represent a unique class of peroxidase substrates in that they can be intrinsically detectable before action of the enzyme but are "fixed in place" by the action of a peroxidase in the process described as tyramide signal amplification (TSA). These substrates are extensively utilized to label targets in samples that are cells, tissues or arrays for their subsequent detection by microscopy, flow cytometry, optical scanning and fluorometry.

Another preferred colorimetric (and in some cases fluorogenic) substrate and enzyme combination uses a phosphatase enzyme such as an acid phosphatase, an alkaline phosphatase or a recombinant version of such a phosphatase in combination with a colorimetric substrate such as 5-bromo-6-chloro-3-indolyl phosphate (BCIP), 6-chloro-3-indolyl phosphate, 5-bromo-6-chloro-3-indolyl phosphate, *p*-nitrophenyl phosphate, or *o*-nitrophenyl phosphate or with a fluorogenic substrate such as 4-methylumbelliferyl phosphate, 6,8-difluoro-7-hydroxy-4-methylcoumarinyl phosphate (DiFMUP, U.S. Pat. No. 5,830,912) fluorescein diphosphate, 3-*O*-methylfluorescein phosphate, resorufin phosphate, *9H*-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl) phosphate (DDAO phosphate), or ELF 97, ELF 39 or related phosphates (U.S. Pat. Nos. 5,316,906 and 5,443,986).

Glycosidases, in particular beta-galactosidase, beta-glucuronidase and beta-glucosidase, are additional suitable enzymes. Appropriate colorimetric substrates include, but are not limited to, 5-bromo-4-chloro-3-indolyl beta-D-galactopyranoside (X-gal) and similar indolyl galactosides, glucosides, and glucuronides, *o*-nitrophenyl beta-D-galactopyranoside (ONPG) and p-nitrophenyl beta-D-galactopyranoside. Preferred fluorogenic substrates include resorufin beta-D-galactopyranoside, fluorescein digalactoside (FDG), fluorescein diglucuronide and their structural variants (U.S. Pat. Nos. 5,208,148; 5,242,805; 5,362,628; 5,576,424 and 5,773,236), 4-methylumbelliferyl beta-D-galactopyranoside, carboxyumbelliferyl beta-D-galactopyranoside and fluorinated coumarin beta-D-galactopyranosides (U.S. Pat. No. 5,830,912).

Additional enzymes include, but are not limited to, hydrolases such as cholinesterases and peptidases, oxidases such as glucose oxidase and cytochrome oxidases, and reductases for which suitable substrates are known.

Enzymes and their appropriate substrates that produce chemiluminescence are preferred for some assays. These include, but are not limited to, natural and recombinant forms of luciferases and aequorins. Chemiluminescence-producing substrates for phosphatases, glycosidases and oxidases such as those containing stable dioxetanes, luminol, isoluminol and acridinium esters are additionally useful.

In addition to enzymes, haptens such as biotin are also preferred labels. Biotin is useful because it can function in an enzyme system to further amplify the detectable signal, and it can function as a tag to be used in affinity chromatography for isolation purposes. For

detection purposes, an enzyme conjugate that has affinity for biotin is used, such as avidin-HRP. Subsequently a peroxidase substrate is added to produce a detectable signal.

Haptens also include hormones, naturally occurring and synthetic drugs, pollutants, allergens, affector molecules, growth factors, chemokines, cytokines, lymphokines, amino acids, peptides, chemical intermediates, nucleotides and the like.

Fluorescent proteins also find use as labels for the labeling reagents of the present invention. Examples of fluorescent proteins include green fluorescent protein (GFP) and the phycobiliproteins and the derivatives thereof. The fluorescent proteins, especially phycobiliprotein, are particularly useful for creating tandem dye labeled labeling reagents. These tandem dyes comprise a fluorescent protein and a fluorophore for the purposes of obtaining a larger stokes shift wherein the emission spectra is farther shifted from the wavelength of the fluorescent protein's absorption spectra. This is particularly advantageous for detecting a low quantity of a target in a sample wherein the emitted fluorescent light is maximally optimized, in other words little to none of the emitted light is reabsorbed by the fluorescent protein. For this to work, the fluorescent protein and fluorophore function as an energy transfer pair wherein the fluorescent protein emits at the wavelength that the fluorophore absorbs at and the fluorphore then emits at a wavelength farther from the fluorescent proteins than could have been obtained with only the fluorescent protein. A particularly useful combination is the phycobiliproteins disclosed in US Patents 4,520,110; 4,859,582; 5,055,556 and the sulforhodamine fluorophores disclosed in 5,798,276, or the sulfonated cyanine fluorophores disclosed in US serial Nos. 09/968/401 and 09/969/853; or the sulfonated xanthene derivatives disclosed in 6,130,101 and those combinations disclosed in US Patent 4,542,104. Alternatively, the fluorophore functions as the energy donor and the fluorescent protein is the energy acceptor.

The labeling reagents can be independently attached to one or more labels of the present invention by a number of methods known to one skilled in the art and modification of such methods. Methods include, labeling in a solution or on an affinity column. For labeling in solution the labeling reagent is optionally modified to contain a reactive group and the label is modified to contain a reactive group or is synthesized to contain a reactive group, as is typically the case with fluorophore labels wherein the reactive group facilitates covalent attachment. The modification of the labeling reagent to contain a reactive group includes (1) chemical addition of such a reactive group or (2) alternatively takes advantage of the disulfide bonds of the $F(ab')_2$ fragment wherein the fragment is reduced to break the bond and expose the thiol group that readily reacts with a reactive group on a label, as disclosed

in US Patent No. 5,360,895. Typically, covalent attachment of the label to the fragment is the result of a chemical reaction between an electrophilic group and a nucleophilic group, *See* Table 1 for list of useful electrophile and nucleophile reactive groups. However, when a label is used that is photoactivated the covalent attachment results when the labeling solution is illuminated.

A method for covalently attaching a label, particularly an enzyme, a fluorescent protein or a particle, comprises the following steps:

a) cleaving an intact anti-region antibody with an enzyme resulting in a $F(ab')_2$ fragment;
b) contacting said $F(ab')_2$ fragment with a reducing agent to produce Fab' fragments containing a thiol group;
c) contacting said Fab' fragments with a solution comprising a label that contains a reactive group; and,
d) isolating Fab' fragments of step d) that are covalently attached to a label by size exclusion or affinity chromatography.

The whole anti-region antibody is cleaved with pepsin to generate a bivalent $F(ab)'_2$ fragment. This fragment is typically affinity purified on a column comprising immunoglobulin proteins such as IgG that is immobilized on agarose. The fragment is then reduced to break the disulfide bond of the hinge region that connects the two Fab fragments resulting in a Fab' fragment with an exposed thiol group. This is typically accomplished by adding a mild reducing buffer to the affinity purified $F(ab')_2$ fragments such as a buffer comprising 0.01 M EDTA and 0.01M cysteine in phosphate buffer saline (PBS). The resulting thiol group readily reacts with a reactive group on a label to covalently attach the label to the fragment. Thus, a solution containing a label that has been chemically modified to contain a reactive group, using methods well known to one skilled in the art, is added to the solution of reduced Fab' fragments. This method is particularly useful for covalently attaching enzyme and other protein labels due to their size and the lack of exposed amine groups on the Fab fragments. One of skill in the art will appreciate that this method requires the use of Fab' fragments as apposed to Fab fragments due to the disulfide bonds of the Fab' fragment and that the use of the enzyme papain or the like results in such a fragment.

An alternative labeling of monovalent antibody fragments and the monomeric non-antibody proteins is also accomplished in a solution. The method comprises the steps:

a) contacting a Fab fragment or non-antibody monomeric protein with a solution comprising a label that contains a reactive group; and,
b) isolating labeled anti-region Fab fragment or non-antibody monomeric protein by size exclusion or affinity chromatography.

When a Fab fragment is to be labeled the whole antibody is cleaved with an enzyme, such as papain, to generate Fab monovalent fragments and the fragments are typically purified on an affinity column prior to addition of the label. The Fab fragment or non-antibody monomeric proteins are optionally chemically modified to contain a reactive group. However, for covalently attaching reactive fluorophore labels it has been found that this modification of the fragment of non-antibody protein is not necessary. The reactive label, typically a fluorophore or hapten, are added to a solution of Fab fragments or non-antibody proteins and the labeling reagent is separated from excess label by size exclusion or affinity chromatorgraphy. The labeling reagents are then stored in an appropriate buffer.

Labeling in solution can have some drawbacks, especially when labeling of Fab fragments or non-antibody proteins with fluorophores. Thus, Fab fragments and non-antibody proteins of the present invention are preferably covalently attached to a fluorophore label when immobilized on an affinity column. The fragments and non-antibody proteins are immobilized on an affinity column that comprises a protein that the fragment has affinity for, typically IgG, and after immobilization a reactive fluorophore is added to the column wherein the fragments are labeled and unreacted fluorophores pass through the column.

The use of this affinity chromatography method avoids the incorporation of label into the binding domain of the Fab fragment or non-antibody protein. When Fab fragments are labeled with fluorophores using this method unexpected advantages were obtained wherein the fluorescent signal form fragments labeled on a column are brighter than fragments labeled in solution when the fluorophore and ratio of fluorophore to labeling reagent are held constant. Without wishing to be bound by a theory it is possible that the decreased brightness observed from the fragments labeled in solution is due to quenching of fluorphores that are bound in or near the binding domain by the high concentration of amine groups in the binding domain. Thus, a preferred embodiment of the invention for covalently attaching fluorphore labels to Fab fragments comprises the following steps:

a) cleaving an intact anti region antibody with an enzyme that generates Fab fragments;
b) isolating the anti-region Fab fragments of step a);

c) contacting a matrix comprising intact immunoglobulin proteins or fragments thereof that specifically bind anti-region Fab fragments with a solution comprising said anti-region fragments of step b) wherein said Fab fragments are immobilized;

d) contacting said matrix of step c) with a solution comprising a fluorophore label that contains a reactive group;

e) washing said matrix to remove unbound label, and;

f) eluting said labeling reagent from said matrix whereby said labeling reagent is manufactured comprising a label and being isolated from other proteins and fragments thereof.

The matrix is typically an agarose column that comprises either the selected region, such as the Fc region, or the entire antibody provided that the antibody or fragment thereof is the same species and isotype that was used to produce the antibodies that the labeling reagent was generated from. However any matrix known to one skilled in the art can be used that allows for immobilization of labeling reagent and removal following attachment of the fluorophore label. Fab and Fab' fragments can both be labeled in this manner. However a free thiol group is not necessary and therefore Fab fragments are typically labeled using this method.

Due to the unique properties of the labeling reagent and the attached labels it is a preferred embodiment of the present invention that enzyme or other protein labels are covalently attached to Fab' fragments in solution utilizing the free thiol group of the Fab' fragment. It is another preferred embodiment that fluorophore labels be covalently attached to the labeling reagent when the reagent is immobilized on a affinity column wherein the labeling reagent is typically an Fab fragment or a non-antibody monomeric protein.

The attachment of the label to the fragments or the non-antibody proteins results in multiple subsets that are distinguished by the ratio of the label to the labeling reagent and the physical properties of the label. A labeling reagent subset as used herein refers to a discrete set of labeling reagents that are homogenous and can be distinguished from another subset of labeling reagent either by the physical properties of the label or the ratio of the label to labeling reagent. The physical properties include differences within a group of labels, such as emission spectra of fluorphores, or across groups of labels, such as the difference between an enzyme and a fluorophore. For fluorphore labels, the physical properties typically relates to the emission spectra, this includes modification of the same label, e.g. a cyanine with different substitutions that shifts the emission wavelength, or different

fluorophores, e.g. a cyanine and a coumarin on the same labeling reagent. The difference in physical properties also includes the use of tandem dyes, which is specifically defined to include an energy transfer pair wherein one is a protein and the other is a fluorophore or both are fluorophores, or the pairing of other labels that are not necessarily energy transfer pairs. A few examples of labeling reagent subsets includes, but are not limited to, a first subset comprising a single fluorophore at a known ration attached to a anti-Fc Fab fragment; a second subset comprises the same fluorophore on the Fab fragment at a different known ration from the first subset, a third subset comprises the same fluorophore but that has a shifted wavelength due to a substitution on the fluorophore. Thus, the attachment of labels to the labeling reagents results in an extensive selection of subsets that when complexed with a target-binding antibody results in a unique method to detect one or multiple targets in a sample whereby the target is identified and quantitated.

C. Immuno-labeled Complex

The subsets of labeling reagent are complexed with target-binding antibodies to produce subsets of immuno-labeled complex that for the target detection solution. The methods for forming the immuno-labeled complex comprises the following steps:

a) contacting a solution of target-binding antibodies with a labeling reagent subset, wherein said labeling reagent subsets are distinguished by i) ratio of label to labeling reagent or ii) a physical properties of said label;
b) incubating said target-binding antibodies and said labeling reagent for a time period sufficient for one or more labeling reagents to form an immuno-labeled complex with a target-binding antibody wherein a region of said target binding antibody is selectively bound by labeling reagent;
c) optionally removing unbound labeling reagent by adding a capture reagent comprising immunoglobulin proteins or fragments thereof; and,
d) optionally repeating said steps a), b), and c) to form individual or pooled subsets of immuno-labeling complexes wherein each subset is distinguished from another subset by i) a ratio of label to labeling reagent, or ii) a physical property of said label, or iii) a ratio of labeling reagent to said target-binding antibody, or iv) by said target-binding antibody.

A particular advantage for the use of labeling reagent of the present invention to label target-binding antibodies is that the process is relatively insensitive to the solution the antibodies

are in. Due to the physical nature of the labeling reagents, small monovalent fragments, the reagents do not cross-link and fall out of solution in the presence of high concentration of proteins. For this reason, target-binding antibodies can be complexed when present in ascites fluid, tissue culture supernatant, serum or other solutions where there is a high concentration of proteins. This eliminates the need to purify target-binding proteins prior to labeling.

When preparing the immuno-labeled complex using purified target-binding antibody, stock solutions of both the labeling reagent and the target-binding antibody are typically near 1 mg/mL in an appropriate buffer, although more or less concentrated solutions are also suitable. Generally, the labeling reagent is mixed in a molar ratio of at least one to 50 moles of labeling reagent to one mole of the target-binding antibody to be complexed. More commonly a ratio of at least one to as many as 10 moles of labeling reagent per mole of target-binding antibody is combined. With an anti-Fc region Fab to a target-binding antibody, a molar ratio of approximately 2 to 10 is typical, more typically 3 to 5 (particularly for complexes in which the labeling reagent has been labeled while immobilized on an affinity matrix). The ease of formation of the complex permits rapid optimization of the complex and assessment of the effect of variation in experimental parameters. A particularly unique advantage of the invention is that the stoichiometry of the complex is easily adjusted to provide complexes with different ratios of labeling reagent to target-binding antibody, and thus there is control over the ultimate detectability of the target in the sample. Complexes that have been labeled with the same dye but at different molar ratios can be separately detected by the differences in their intensities.

Complex formation appears to occur almost within the mixing time of the solutions (< 1 minute) but the reaction typically is allowed to proceed for at least 5 minutes and can be longer before combining the immuno-labeled complex with the sample. Although complex formation can be reversed by addition of an unlabeled antibody that contains the same binding region, reversibility is very slow; furthermore, following binding of the immuno-labeled complex to a target in a sample, the sample can be "fixed" using aldehyde-based fixatives by methods that are commonly practiced by those skilled in the art of immunolabeling.

The labeling process optionally further comprises the addition of a capture component to remove excess labeling reagent. For applications in which immunolabeling complexes of multiple primary antibodies from the same species (e.g. mouse monoclonal antibodies) or cross-reacting species (e.g. mouse and human antibodies) are to be used simultaneously or

sequentially, it is necessary to quench or otherwise remove any excess labeling reagent by use of a capture component or by other means to avoid inappropriate labeling of the sample. The most effective capturing components to capture excess labeling reagent are those that contain the binding site of the labeling reagent but are themselves not labeled, preferably an antibody or antibody fragment. Capture components may be free in solution or immobilized on a matrix, such as agarose, cellulose, or a natural or synthetic polymer, to facilitate separation of the excess capture component from the immuno-labeled complex. The capture component is optionally attached to a microsphere or magnetic particle. However, separation of excess labeling reagent is not essential for successful utilization of the invention, particularly when using a single target-binding antibody.

The steps of the labeling process for the target-binding antibodies can be repeated to form discrete immuno-labeled complex subsets that can be used individually or pooled in an assay to detect individual or multiple targets. As used herein the term immuno-labeled complex subsets refers to subsets that are distinguished from each other i) a ratio of label to labeling reagent, or ii) a physical property of the label, or iii) a ratio of labeling reagent to the target-binding antibody, or iv) by the target-binding antibody, or a combination thereof. For example a panel of subsets may comprise a target-binding antibody that is bound by a labeling reagent comprising a subset of different ratios of the same label on the labeling reagent resulting in a discrete subset of immuno-labeled complexes. This subset of immuno-labeled complexes can be used individually wherein a target is identified by the intensity of the detectable label or used in combination with another subset of immunocomplexes that differ in the target-binding antibody to identify multiple targets.

C. Ligand-detection Reagent

The immuno-labeled complexes can be modified by the addition of a ligand analog of the present invention to form a ligand-detection reagent. The present ligand-detection reagents comprise a ligand-binding antibody, a ligand analog and a labeling reagent, *See* Figure 8. This ligand-detection reagent is formed by incubating the ligand-binding antibody, the ligand analog and the labeling reagent for sufficient amount of time to allow for a complex to form. The formation of the complex happens fairly rapidly, typically less than 30 minutes, preferably less than 15 minutes and most preferred the complex forms in 5 minutes or less.

The reporter molecules that are covalently attached to the ligand analog are preferably selected from the group consisting of a borapolyazaindacene, a coumarin, a xanthene, a cyanine, a fluorescent protein and a phosphorescent dye. Most preferred are

borapolyazaindacene, fluorinated xanthene, fluorinated coumarin, including dyes sold under the trade name OREGON GREEN, BODIPY, PACIFIC BLUE and MARINA BLUE (Trade marks owned by Molecular probes, Inc.) including any dyes disclosed in US Patent Nos. 6,162,931; 5,830,912; 4,774,339; 5,187,288; 5,248,782; and 5,433,896.

Ligand analogs containing these preferred reporter molecules were screened with ligand-binding antibodies specific for phosphotyrosine. Thus, preferred ligand-detection reagents for this application include a phosphotyrosine-binding antibody and the phosphotyramide and phosphotyrosinamide ligand analogs. Preferred ligand analogs include Compounds **6-38**. Most preferred are Compounds **7, 9, 12, 15, 19, 23, 25, 27, 33, 34** and **38.** When the ligand-detection reagent comprise Compound **9** or **15** the respective reporter group is quenched by 80% or more.

Thus, a preferred embodiment of the present invention includes a ligand-detection reagent that comprises a ligand-binding antibody, a ligand analog and a labeling reagent to form an antibody-ligand analog-labeling reagent complex wherein said ligand analog is selected from the group consisting of phosphotyramide, phosphotyrosinamide, phosphoethanolamine, phosphorylated kinase peptide substrate, phosphatase substrate and phosphorylated peptide and said analog is covalently bonded to a xanthene or borapolyazaindacene reporter molecule and the reporter molecule of ligand analog is capable of being quenched when bound by said ligand-binding antibody. Preferably, the reporter molecule is quenched by about 80% or more. The labeling reagent comprises a label that is a fluorophore or a quenching moiety and a monovalent antibody fragment or a non-antibody protein wherein the label functions as an energy acceptor molecule. The labeling reagent is incubated with the ligand-binding antibody and the ligand analog for a sufficient amount of time to form a ligand-detection reagent.

In another aspect of the invention, fluorogenic ligand analogs are used resulting in fluorogenic ligand-detection reagents. In this instance, the ligand-detection reagent is fluorescent but when the target ligand displaces the ligand analog the fluorescent signal intensity deceases. Thus, the presence of a target ligand is determined by a decrease in fluorescence signal. Preferred fluorogenic ligand analogs include Compounds **4** and **5**.

When preparing the ligand-detection reagent with a labeling reagent, the complex is formed as disclosed above for the immuno-complexes. The ligand analog is added before, with or after the addition of the labeling reagent to the ligand-binding antibody. The entire complex forms very rapidly, typically less than 5 minutes.

Appropriate matching of the reporter group and label are necessary to maximize the FRET between the reporter molecule and label for either optimal quenching or emission of energy at a longer wavelength. Many energy transfer dye pairs are known to one of skill in the art. Table 2 lists representative energy transfer pair dyes wherein the acceptor functions as a quenching moiety. This list is not intended to be limiting.

Table 2:

Donor Dye	Acceptor Compounds
Alexa Fluor 350	Alexa Fluor 488; QSY 36; dabcyl
Alexa Fluor 488	Alexa Fluor 546; Alexa Fluor 555; Alexa Fluor 568; Alexa Fluor 594; Alexa Fluor 647; QSY 35; Dabcyl; QSY 7; QSY 9
Alexa Fluor 546	Alexa Fluor 568; Alexa Fluor 594; Alexa Fluor 647; QSY 35; Dabcyl; QSY 7; QSY 9
Alexa Fluor 555	Alexa Fluor 594; Alexa Fluor 647; QSY 7; QSY 9
Alexa Fluor 568	QSY 7; QSY 9; QSY 21
Alexa Fluor 594	Alexa Fluor 647;

	QSY 21
Alexa Fluor 647	QSY 21
Fluorescein	Tetramethylrhodamine; QSY 7; QSY 9
IAEDANS	Fluorescein
BODIPY FL	Alexa Fluor 555; QSY 9

Preferred energy transfer dye pairs are selected from the group consisting of Oregon Green 488-Alexa Fluor 555 dye pair, BODIPY-FL-Alexa Fluor 555 dye pair and BODIPY-FL-QSY 9 dye pair.

Therefore, a preferred embodiment of the present invention includes a ligand-detection reagent that comprises a ligand antibody, a ligand analog and a labeling reagent to form a ternary complex wherein said ligand analog is selected from the group consisting of phosphotyramide, phosphoethanolamine, phosphorylated kinase peptide substrate, phosphatase substrate and phosphorylated peptide and said analog is covalently bonded to a xanthene reporter molecule and said labeling reagent is an anti-Fc monovalent antibody fragment covalently bonded to a xanthene label moiety or non-fluorescent quenching moiety.

For the detection of phosphorylated molecules and enzymes that modify the degree of phosphorylation the phosphotyramide, phosphotyrosinamide, phosphoserine and phosphoethanolamine ligand analogs are preferred, including Compounds **2** and **4-43**. In addition, proteins or peptides that have been modified to be a ligand analog are also preferred. Table 3 contains a select list of some peptides that are specific for phosphotyrosine-binding antibodies that when conjugated to a reporter molecule of the present invention forms a ligand analog.

Table 3: phosphotyrosine ligands

Peptide	**Sequence**
pY-1	ENDpYINASL
pY-2	DADEpYLIPQQG
EGF Receptor	DADEpYL
M-2170	IpYGEF
M-2165	IYGEF
M-2035	TEPEpYQPGE
N-1480	DpYVPML
H-1546	Biotin-EPQpYEEIPIYL

H-5458	Biotin-EGPWLEEEEEAYGWMSF
pp60	TSTEPQpYQPGENL
abl peptide	EAIYAAPFAKKK
DSIP	WAGGDASGE
pDSIP	WAGGDApSGE
pY	pY

It is appreciated that the ligand-detection reagents can be designed to detect an unlimited number of target ligands utilizing a ligand analog and an appropriately matched ligand-binding antibody and that these reagents are in no way limited to the detection of phosphorylated biomolecules. Thus, ligand analogs and or target ligands are preferably selected from the group consisting of an amino acid, an enzyme, a kinase substrate, a peptide, a protein, a polysaccharide, a phosphatase substrate, a nucleoside, a nucleotide, an oligonucleotide, a nucleic acid, a hapten, digoxigenin, a cell surface receptor, a drug, a hormone, a lipid, a lipid assembly, a synthetic polymer, a polymeric microparticle, a biological cell and a virus wherein the ligand analog further comprise a reporter molecule. Preferred ligand analogs for the detection of phosphorylated biomolecules are selected from the group consisting of phosphotyramide, phosphotyrosinamide, phosphoethanolamine, phosphorylated kinase peptide substrate, phosphatase substrate and phosphorylated peptide. These ligand analogs are preferably conjugated to xanthene, borapolyazaindacene or coumarin reporter molecules.

C. Methods of Use

The labeling reagents, target-binding antibodies and resulting immuno-labeled complex that forms the target detection solution can be used in a wide range of immunoassays, essentially in any assay a traditional secondary antibody is used including some assays that secondary antibodies are not used because of their size and ability to cross-link. Examples of such assays used to detect a target in a sample include immunoblots, direct detection in a gel, flow cytometry, immunohistochemistry, confocal microscopy, fluorometry, ELISA and other modified immunoassays. Furthermore, the immuno-labeled complex can be modified by the addition of a ligand analog to form a ligand-detection reagent complex. In this instance, the ligand-detection reagent is employed in a competitive immunoassay wherein a target ligand displaces the ligand analog resulting in a change in detectable signal that indicates the presence of a target ligand in a sample.

A method of the present invention for detecting a single target in a sample comprises the following steps:

a) contacting a solution of target-binding antibodies with a labeling reagent subset, wherein said labeling reagent subsets are distinguished by i) ratio of label to labeling reagent or ii) a physical properties of said label;

b) incubating said target-binding antibodies and said labeling reagent subset for a time period sufficient for one or more labeling reagents to form an immuno-labeled complex with a target-binding antibody wherein a region of said target binding antibody is selectively bound by labeling reagent;

c) contacting said sample with said immuno-labeled complex of step b);

d) incubating said sample of step c) for a time sufficient to allow said immuno-labeled complex to selectively bind to said target; and,

e) illuminating said immuno-labeled complex whereby said target is detected.

A sample is incubated with a preformed immuno-labeled complex that comprises a labeling reagent and a target-binding antibody. While this method describes the identification of a single target, subsets of labeling reagents bound to the same target-binding antibody can be used to identify and provide additional information about such targets. For example, subsets of labeling reagent can be prepared wherein two discrete subsets are generate each with a distinct fluorophore label that is distinguished by their emission spectra, e.g. one that emits in the green spectra and one that emits in the red spectra. The labeling reagent subsets are then added to a solution of target-binding antibody in a controlled ratio, e.g. two parts one labeling reagent (green emission) and one part the other labeling reagent (red emission) per target binding antibody. In this way the immuno-labeled complexes can be used to detect a target. If another immuno-labeled complex were added to the sample the original target could be distinguished from the subsequently detected target.

The methods of the present invention also provide for the detection of multiple targets in a sample. Multiple targets include the discrete epitope that the target-binding antibody has affinity for as well as molecules or structures that the epitiope is bound to. Thus, multiple target identification includes phenotyping of cells based on the concentration of the same cell surface marker on different cells. In this way multiple target identification is not limited to the discrete epitope that the target binding antibody binds, although this is clearly a way that multiple targets can be identified, i.e. based on the affinity of the target-binding antibody.

Therefore, a method for detecting multiple targets in a sample comprises the following steps:

a) contacting a solution of target-binding antibodies with a labeling reagent subset, wherein said labeling reagent subsets are distinguished by i) ratio of label to labeling reagent or ii) a physical properties of said label;

b) incubating said target-binding antibodies and said labeling reagent subset for a time period sufficient for one or more labeling reagents to form an immuno-labeled complex with a target-binding antibody wherein a region of said target-binding antibody is selectively bound by labeling reagent, wherein steps a) and b) are repeated to form discrete immuno-labeling complex subsets;

c) contacting said sample with a solution comprising A) a pooled subset of immuno-labeled complexes, wherein each subset is distinguished from another subset by i) a ratio of label to labeling reagent, or ii) a physical property of said label, or iii) a ratio of labeling reagent to said target-binding antibody, or iv) by said target-binding antibody or B) an individual subset wherein step c) with a solution comprising an individual subset is repeated;

d) incubating said sample of step c) for a time sufficient to allow said immuno-labeled complex to selectively bind to said target; and,

e) illuminating said immuno-labeled complex whereby said target is detected.

A selected target-binding antibody and a subset of labeling reagent are incubated to form an immuno-labeled complex subset. This procedure is repeated to form a panel of immuno-labeled complex subsets that may be pooled and added to a sample. Alternatively each immuno-labeled complex subset is added stepwise to a sample. The immuno-labeled complex subsets are distinguished by four characteristics resulting in an infinite number of immuno-labeled complex subsets. First (i) the subsets can be distinguished by the target-binding antibody that is determined by the end user for the information that is desired from a sample. This means that each subset is distinguished based on the affinity of the target-binding antibody. The target-binding antibody typically distinguishes immuno-labeled complexes when multiple targets are identified, however this is normally combined with another characteristic to gain information form a sample or increase the number of targets that can be detected at one time. The second (ii) distinguishing feature used is the ratio of label to labeling reagent, as discussed in detail above. A subset based on this feature would have for example a ratio of two fluorophore per each labeling reagent. The third (iii) distinguishing feature is the ratio of labeling reagent to target-binding antibody. This is accomplished using a controlled concentration of target-binding antibody mixed with a controlled concentration of a labeling reagent subset and the subset would comprise a target-binding antibody that is bound by a discrete number of labeling reagents. The fourth (iv) feature is the physical feature of the label. Typically this refers to the physical properties

of the fluorophore labels wherein a subset of this group is distinguished by the label itself such as a green emitting fluorophore compared to a red emitting fluorophore. One of skill in the art will appreciate that while immuno-labeling complex subsets can be distinguished based on one feature the subsets are typically, and most useful, when discretely identified based on a combination of the distinguishing characteristics.

Another example of detection of multiple targets utilizes the following immuno-labeled subsets, all of which comprise a different target-binding antibody but differ in the label and ratio of label. The first subset comprises a fluorophore label that emits red-fluorescent light, a second subset comprises a fluorophore label that emits green fluorescent light, a third subset comprises a ratio of 1:1 red to green fluorophore label; a fourth subset comprises a ratio of 2:1 red to green fluorophore label and a fifth subset comprises a ratio of 1:2 red to green fluorophore label. These subsets allow for the simultaneous detection of five targets in a sample. This aspect of the present invention is particularly important due to the limited range of fluorophores available wherein the labeling reagents can be utilized to increase the number of targets that can be detected at one time. One of skill in the art can appreciate that these subsets could be expanded by altering the ratio of label to labeling reagent instead of just the ratio of labeling reagent to target-binding antibody. This same methodology can also be applied to a single fluorophore label wherein the ratios are altered and a target is detected based on the intensity of the signal instead of the color and the ratio of the color to another color.

Following the formation of the immuno-labeled complex subsets the subsets can be pooled and added to a sample or added stepwise to a sample, either of which is determined by the end user and the particular assay format. This method of the present invention provides for maximum flexibility and ease of determining multiple targets in a sample.

Another method of the present invention provides for the determination of multiple targets in a sample specifically using the flow cytometry assay format. Traditionally targets identified using flow cytometry used either directly labeled primary antibody or labeled microspheres that were covalently attached to a primary antibody wherein the microsphere is the label. Examples include the fluorescent encapsulated microsphere beads sold by Luminex. The labeling reagents and the present invention overcome both the need for directly labeled primary antibody and the need for expensive microspheres.

Thus, a method of the present invention for determining identity and quantity of targets in a sample by detecting multiple targets comprises the following steps:

a) contacting a solution of target-binding antibodies with a labeling reagent subset, wherein said labeling reagent subsets are distinguished by i) ratio of label to labeling reagent or ii) a physical properties of said label;

b) incubating said target-binding antibodies and said labeling reagent for a time period sufficient for one or more labeling reagents to form an immuno-labeled complex with a target-binding antibody wherein a region of said target binding antibody is selectively bound by labeling reagent, wherein steps a) and b) are repeated to form a pooled subset of immuno-labeling complexes;

c) contacting a population of cells in a sample with a solution comprising a pooled subset of immuno-labeled complexes, wherein each subset is distinguished from another subset by i) a ratio of label to labeling reagent, or ii) a physical property of said label, or iii) a ratio of labeling reagent to said target-binding antibody, or iv) by said target-binding antibody;

d) incubating said cells for a time period sufficient to allow said immuno-labeled complex to bind said targets;

e) passing said incubated population of cells through an examination zone; and,

f) collecting data from said cells that were passed through said examination zone wherein said multiple targets are detected whereby the identity and quantity of said targets is determined.

In one aspect, a target-binding antibody is pre-complexed to the target-binding antibody to form a subset and that subset or a panel of subsets are added to a sample, that are typically distinguished by the target binding antibody. This method then avoids the need for a directly labeled primary. Secondly, when the panel of subsets is distinguished, for example, by the ratio of label to labeling reagent or the ratio of labeling reagent to target-binding antibody the immuno-labeled complex can function similar to the microsphere beads of Luminex. For example, this is accomplished wherein three immuno-labeled complex subsets are distinguished by the target binding antibody and the fluorophore attached to the labeling reagent and within one of the subsets is another set of subsets that are distinguished based on the ratio of label to labeling reagent. In this way three different epitopes are detected and one of the epitopes is further distinguished and a phenotype distinction made based on the intensity of the signal generated from the labeled-immuno complex subsets based on the ratio of fluorophore to labeling reagent. This determination of targets is facilitated when a population of cells or cellular organelles is passed through the examination zone of a flow cytometer wherein the fluorescent signal and intensity is recorded for each cell resulting in a histogram of the cell population or cellular organelles based on the detected epitopes.

In another aspect of the invention, additional detection reagents are combined with the sample concurrently with or following the addition of immuno-labeled complex subsets. Such additional detection reagents include, but are not limited to reagents that selectively detect cells or subcellular components, ions, or indicate the cell viability, life cycle, or proliferation state. For example, the additional detection reagent is a labeled target-binding antibody that is directly or indirectly detectable and another additional detection reagent is a stain for nucleic acids, for F-actin, or for a cellular organelle.

In another aspect of the invention the immuno-labeled complexes are modified to contain a ligand analog wherein the resulting ternary complex is utilized as a ligand-detection reagent in a competitive immunoassay. The ligand-detection reagents of the present invention can be used without limitation for the detection, analysis and monitoring of target ligands. These ligand-detection reagents are typically present in a ligand-detection solution, wherein the solution comprises a ligand antibody, a ligand analog and a labeling reagent to form a ternary ligand-detection reagent complex and a buffer. Appropriate buffers include the family of Good's buffers or any buffer known to one of skill in the art that is typically used with antibodies.

Therefore, in one aspect of the invention a method for determining the presence of a target ligand in a sample comprises

a) generating a ligand-detection reagent, wherein the ligand-binding antibody, the ligand analog and the labeling reagent are incubated together for a sufficient amount of time to form the ligand-detection reagent;

b) incubating the reagent with said sample for a sufficient amount of time for said ligand to displace said ligand analog from binding groove of said ligand-binding antibody;

c) illuminating said sample with an appropriate wavelength wherein said reporter molecule generates a detectable signal in the presence of said ligand whereby said ligand is detected.

The ligand-detection reagent is generated as described above. The reagent is incubated with the sample for a sufficient amount of time for the target ligand to displace the ligand analog. Typically this occurs very rapidly, usually within 5 minutes or less, preferably the displacement occurs within seconds of adding the ligand-detection reagent to the sample, See, Example 34. Illumination of the reporter molecule, as described below, depends on the reporter molecule of the ligand analog.

It is envisioned that any target ligand, wherein an appropriate ligand-binding antibody exists, can be detected using this method of the present invention. This includes the use of either a fluorogenic or non-fluorogenic ligand analog and a labeling reagent wherein the labeling reagent functions to quenching the reporter molecule when bound by the ligand-binding antibody or to further shift the detectable signal from the reporter molecule signal.

In one aspect of the invention, the method for determining the presence of a target ligand in a sample is used to detect phosphorylated biomolecules. In this instance the ligand-binding solution typically comprises a ligand-binding antibody that is capable of binding a phosphotyrosine, phosphoserine or phosphothreonine moiety, an appropriately matched ligand analog that is selected from the group consisting of phosphotyramide, phosphoserine phosphotyrosinamide, phosphoethanolamine, phosphorylated kinase peptide substrate, phosphatase substrate and phosphorylated peptide, and optionally a labeling reagent whereby the amount of generated detectable signal from said reporter molecule is dependent on the presence of the phosphorylated target ligand. Preferably, the ligand analog is covalently bonded to a xanthene, coumarin, or borapolyazaindacene reporter molecule and said labeling reagent is an anti-Fc monovalent antibody fragment covalently bonded to a xanthene labeling moiety or non-fluorescent quenching moiety.

Incubating phospho –tyrosine, -threonine or -serine binding antibodies with an appropriately matched ligand analog and labeling reagent, generates the ligand-detection reagent. Preferably the ligand analog comprises a phosphophenol moiety including both phosphotyramide and phosphotyrosinamide ligand analogs. The labeling reagent may be added prior to the addition of the ligand analog, after the addition of the ligand analog or all three components may be added simultaneously to form a ligand-detection reagent.

Following formation of the ligand-detection reagent in a ligand-detection solution, which comprises the reagent and an appropriate buffer, the ligand-detection reagent is incubated with the sample. If present, the target ligand will displace the ligand analog from the ligand-binding antibody almost immediately, preferably less than 5 minutes. The ligand-detection reagent may be illuminated with an appropriate wavelength, before, during or after the reagent has been added to the sample. Alternatively, the reagent may be illuminated continuously from the time of formation to a time point after the reagent has been added to the sample.

This particular method also allows for the detection of enzymes that modify phosphorylated biomolecules, such as kinase and phosphatase enzymes, See Example 39.

Current commercial kinase and phosphatase assays are often time-consuming and require many steps such as electrophoresis, centrifugation, ELISA or immunoprecipitation. The present invention provides methods for the rapid, sensitive, and non-radioactive detection of a variety of selected kinases and phosphatases and provides, in addition, methods that are well suited for high-throughput screening. The kinase and phosphatase assays of the present invention also permit the screening of inhibitors and activators of, for example, tyrosine kinases and, in addition, also permit the monitoring and the purification of kinase and phosphatase enzymes. The enzyme substrate may be on a solid-or semi solid matrix such as an array including Hydrogel slides or present in a solution. The methods of the present invention are particularly advantageous for the monitoring of kinase and phosphatase activity in solution wherein the additional step adding the substrate to a matrix is not necessary. After the formation of the ligand-detection reagent the reagent is added along with enzyme substrate and enzyme to an appropriate buffer, such as kinase buffer comprising 50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 0.01% Brij 35, 2mM DTT and 500 μM ATP. The sample is typically continually illuminated and monitored or at set intervals for a period of time to determine the presence of kinase activity by the addition of phosphate groups to tyrosine, threonine or serine residues on an appropriate substrate. We have demonstrated that ATP does not displace the phosphotyramide ligand analog and therefore the observed detectable signal is related directly to the displacement of the ligand analog by the phosphorylated enzyme substrate, See Example 38.

In addition to a solution based assay, the present methods are also preferred for assay systems that employ immobilized enzyme substrate. In this instance, detection of the enzyme substrate on the array makes the methods of the invention far more sensitive than any known assays for kinases and phosphatases and use of fluorescence for detection on the array permits a higher density of labeling than is possible with radiochemical detection.

In addition to detecting a target ligand as an end point, the present ligand-detection reagents and methods for determining the presence of a target ligand in a sample can be employed to detect and monitor enzymes that directly or indirectly modify the target ligand.

The sample to be used with the present methods and compositions is defined to include any material that may contain a target to which an antibody has affinity for. Typically the sample is biological in origin and comprises tissue, cell or a population of cells, cell extracts, cell

homogenates, purified or reconstituted proteins, recombinant proteins, bodily and other biological fluids, viruses or viral particles, prions, subcellular components, or synthesized proteins. Possible sources of cellular material used to prepare the sample of the invention include without limitation plants, animals, fungi, bacteria, archae, or cell lines derived from such organisms. The sample can be a biological fluid such as whole blood, plasma, serum, nasal secretions, sputum, saliva, urine, sweat, transdermal exudates, cerebrospinal fluid, or the like. Alternatively, the sample may be whole organs, tissue or cells from an animal. Examples of sources of such samples include muscle, eye, skin, gonads, lymph nodes, heart, brain, lung, liver, kidney, spleen, solid tumors, macrophages, mesothelium, and the like.

Prior to combination with the immuno-labeled complexes or ligand-detection reagents, the sample is prepared in a way that makes the target (ligand), which is determined by the end user, in the sample accessible to the immuno-labeled complexes or ligand-binding antibody. Typically, the samples used in the invention are comprised of tissue, cells, cell extracts, cell homogenates, purified or reconstituted proteins, recombinant proteins, biological fluids, or synthesized proteins. Large macromolecules such as immuno-labeled complexes tend to be impermeant to membranes of live biological cells. Treatments that permeabilize the plasma membrane, such as electroporation, shock treatments, or high extracellular ATP, can be used to introduce the immuno-labeled complexes into cells. Alternatively, the immuno-labeled complexes or ligand-detection reagents can be physically inserted into cells, e.g. by pressure microinjection, scrape loading, patch-clamp methods, or phagocytosis. However, the desired target (ligand) may require purification or separation prior to addition of the immuno-labeled complexes or ligand-detection reagent, which will depend on the way the antigenic determinants are contained in the sample. For example, when the sample is to be separated on a SDS-polyacrylamide gel the sample is first equilibrated in an appropriate buffer, such as a SDS-sample buffer containing Tris, glycerol, DTT, SDS, and bromophenol blue.

When the sample contains purified target materials, the purified target materials may still be mixtures of different materials. For example, purified protein or nucleic acid mixtures may contain several different proteins or nucleic acids. Alternatively, the purified target materials may be electrophoresed on gels such as agarose or polyacrylamide gels to provide individual species of target materials that may be subsequently blotted onto a polymeric membrane or detected within the gel matrix. Preparation of a sample containing purified nucleic acids or proteins generally includes denaturation and neutralization. DNA may be denatured by incubation with base (such as sodium hydroxide) or heat. RNA is also

denatured by heating (for dot blots) or by electrophoresing in the presence of denaturants such as urea, glyoxal, or formaldehyde, rather than through exposure to base (for Northern blots). Proteins are denatured by heating in combination with incubation or electrophoresis in the presence of detergents such as sodium dodecyl sulfate. The nucleic acids are then neutralized by the addition of an acid (e.g., hydrochloric acid), chilling, or addition of buffer (e.g., Tris, phosphate or citrate buffer), as appropriate.

Preferably, the preparation of a sample containing purified target (ligand) materials further comprises immobilization of the target materials on a solid or semi-solid support. Purified nucleic acids are generally spotted onto filter membranes such as nitrocellulose filters or nylon membranes in the presence of appropriate salts (such as sodium chloride or ammonium acetate) for DNA spot blots. Alternatively, the purified nucleic acids are transferred to nitrocellulose filters by capillary blotting or electroblotting under appropriate buffer conditions (for Northern or Southern blots). To permanently bind nucleic acids to the filter membranes, standard cross-linking techniques are used (for example, nitrocellulose filters are baked at 80^{o}C in vacuum; nylon membranes are subjected to illumination with 360 nm light). The filter membranes are then incubated with solutions designed to prevent nonspecific binding of the nucleic acid probe (such as BSA, casein hydrolysate, single-stranded nucleic acids from a species not related to the probe, etc.) and hybridized to probes in a similar solution. Purified proteins are generally spotted onto nitrocellulose or nylon filter membranes after heat and/or detergent denaturation. Alternatively, the purified proteins are transferred to filter membranes by capillary blotting or electroblotting under appropriate buffer conditions (for Western blots). Nonspecifically bound probe is washed from the filters with a solution such as saline–citrate or phosphate buffer. Filters are again blocked, to prevent nonspecific adherence of immuno-labeled complexes. Finally, samples are mixed with immuno-labeled complexes or ligand-detection reagents. Nonspecifically bound immuno-labeled complexes or ligand-binding antibodies are typically removed by washing.

When the sample contains cellular nucleic acids (such as chromosomal or plasmid-borne genes within cells, RNA or DNA viruses or mycoplasma infecting cells, or intracellular RNA) or proteins, preparation of the sample involves lysing or permeabilizing the cell, in addition to the denaturation and neutralization already described. Cells are lysed by exposure to agents such as detergent (for example sodium dodecyl sulfate, Tween, sarkosyl, or Triton), lysozyme, base (for example sodium, lithium, or potassium hydroxide), chloroform, or heat. Cells are permeabilized by conventional methods, such as by formaldehyde in buffer.

As with samples containing purified target (ligand) materials, preparation of the sample containing cellular target materials typically further comprises immobilization of the target materials on a surface such as a solid or semi-solid matrix. The targets may be arrayed on the support in a regular pattern or randomly. These supports include such materials as slides, polymeric beads including latex, optical fibers, and membranes. The beads are preferably fluorescent or nonfluorescent polystyrene, the slides and optical fibers are preferably glass or plastic, and the membrane is preferably poly(vinylidene difluoride) or nitrocellulose. Thus, for example, when the sample contains lysed cells, cells in suspension are spotted onto or filtered through nitrocellulose or nylon membranes, or colonies of cells are grown directly on membranes that are in contact with appropriate growth media, and the cellular components, such as proteins and nucleic acids, are permanently bound to filters as described above. Permeabilized cells are typically fixed on microscope slides with known techniques used for *in situ* hybridization and hybridization to chromosome "squashes" and "spreads," (e.g., with a reagent such as formaldehyde in a buffered solution). Alternatively, the samples used may be in a gel or solution.

In a particular aspect of the invention, the sample comprises of cells in a fluid, such as ascites, hybridoma supernatant, or serum, wherein the presence or absence of the target in such cells is detected by using an automated instrument that sorts cells according to the detectable fluorescence response of the detectable moieties in the immunolabeling complexes bound to such cells, such as by fluorescence activated cell sorting (FACS). For methods using flow cytometry a cell population typically comprises individually isolated cells that have been isolated from other proteins and connective tissue by means well known in the art. For example, lymphocyte cells are isolated from blood using centrifugation and a density gradient. The cells are washed and pelleted and the labeling solution added to the pelleted cells.

At any time after addition of the immuno-labeled complex or ligand-detection reagent to the sample, the sample is illuminated with a wavelength of light selected to give a detectable optical response, and observed with a means for detecting the optical response. Equipment that is useful for illuminating the reporter molecule and/or label of the present invention includes, but is not limited to, hand-held ultraviolet lamps, mercury arc lamps, xenon lamps, lasers and laser diodes. These illumination sources are optically integrated into laser scanners, fluorescent microplate readers or standard or microfluorometers. The degree and/or location of signal, compared with a standard or expected response, indicates whether and to what degree the sample possesses a given characteristic, i.e. desired target.

The optical response is optionally detected by visual inspection, or by use of any of the following devices: CCD camera, video camera, photographic film, laser-scanning devices, fluorometers, photodiodes, quantum counters, epifluorescence microscopes, scanning microscopes, flow cytometers, fluorescence microplate readers, or by means for amplifying the signal such as photomultiplier tubes. Where the sample is examined using a flow cytometer, examination of the sample optionally includes sorting portions of the sample according to their fluorescence response.

When an indirectly detectable label is used then the step of illuminating typically includes the addition of a reagent that facilitates a detectable signal such as colorimetric enzyme substrate. Radioisotopes are also considered indirectly detectable wherein an additional reagent is not required but instead the radioisotope must be exposed to X-ray film or some other mechanism for recording and measuring the radioisotope signal. This can also be true for some chemiluminescent signals that are best observed after expose to film.

III. KITS OF THE INVENTION

Suitable kits for preparing an immuno-labeled complex or ligand-detection reagent and for detection of a target (ligand) in a sample also form part of the invention. Such kits can be prepared from readily available materials and reagents and can come in a variety of embodiments. The contents of the kit will depend on the design of the assay protocol or reagent for detection or measurement. Generally, the kits will contain instructions, appropriate reagents and labels, and solid supports, as needed. Typically, instructions include a tangible expression describing the reagent concentration or at least one assay method parameter such as the relative amounts of reagent and sample to be admixed, maintenance time periods for reagent/sample admixtures, temperature, buffer conditions and the like to allow the user to carry out any one of the methods or preparations described above.

A preferred kit of the present invention comprises: a) a labeling solution comprising a labeling reagent that is independently attached to one or more labels and b) a solution comprising a capture reagent. A preferred embodiment of this kit provides a labeling reagent that is anti-Fc Fab fragment, protein G or protein G complexed with albumin. In a more particular embodiment of this kit, the capture component is purified mouse IgG or non-immune mouse serum and the albumin is human albumin, bovine serum albumin, or ovalbumin. In a more preferred embodiment the albumin is ovalbumin. The labeling solution

is either a homogenous mixture of labeling reagents or comprises a pooled subset of labeling reagents. Alternatively the kit comprises a panel of labeling reagent subsets that can be used to make a subset of immuno-labeled complexes.

Additionally the kits may comprise one or more additional components that include (a) stains for characterization of cellular organelles, cell viability, or cell proliferation state, (b) enzyme substrates or (c) enzyme conjugates such as avidin-HRP.

A wide variety of kits and components can be prepared according to the present invention, depending upon the intended user of the kit and the particular needs of the user. It is understood by one skilled in the art, that any of the labeling reagents contemplated by the present invention can be used to in a labeling solution to be included in a kit. The labeling reagents are not intended to be limited to only the described preferred embodiments.

In one aspect of invention a kit for the detection of a target ligand comprises a ligand analog, a labeling reagent and optionally a ligand-binding antibody. In a preferred embodiment the kit comprises a labeling reagent is preferably anti-Fc Fab fragment or anti-kappa Fab fragment and is bound to a fluorophore or quenching moiety. In another embodiment the kit comprises a ligand-binding antibody that has affinity for a phosphorylated biomolecule and an appropriately matched ligand analog that is selected from the group consisting of a phosphotyramide, phosphoserine, a phosphotyrosinamide, a phosphoethanolamine, a phosphorylated kinase peptide substrate, a phosphatase substrate, or a phosphorylated peptide.

A wide variety of kits and components can be prepared according to the present invention, depending upon the intended user of the kit and the particular needs of the user.

IV. APPLICATIONS

The instant invention has useful applications in basic research, high-throughput screening, immunohistochemistry, fluorescence *in situ* hybridization (FISH), microarray technology, flow cytometry, diagnostics, and medical therapeutics. The invention can be used in a variety of assay formats for diagnostic applications in the disciplines of microbiology, immunology, hematology and blood transfusion, tissue pathology, forensic pathology, and veterinary pathology. The invention is particularly useful in the characterization and selection of optimized antibodies from hybridoma supernatants. Additionally, the invention can be used to deliver therapeutics to a specific target. In general, the current invention provides a

versatile and convenient method to enhance any assay that uses an antibody as part of its detection methodology.

The instant invention can be used to study biological phenomena, such as, for example, cell proliferation, signal transduction in cells, or apoptosis. For illustration purposes only and not limitation, one could study thymidine analog 5-bromo-2'-deoxyuridine (BrdU) incorporation. BrdU is a marker for both cell proliferation and apoptosis, as it is readily incorporated into newly synthesized DNA that has progressed through the S-phase of the cell cycle and also into DNA break sites by deoxynucleotidyl transferase (TdT). Anti-BrdU antibodies are used to detect cells marked by BrdU incorporation. By being able to directly label the anti-BrdU antibodies, the current invention provides a convenient method to allow for detection of the incorporated BrdU by conventional immunohistochemistry or fluorescence, depending on detection method required.

Additionally, the current invention has the advantage of allowing staining for multiple targets in one cocktail, thereby reducing the need for more samples or processing steps per experiment. This is particularly important when analyzing precious samples (e.g., pediatric samples, leukocytes isolated from biopsies, rare antigen–specific lymphocytes and mouse tissues that yield a small number of cells). Although it is currently possible to simultaneously measure up to 11 distinct fluorescent colors through a convoluted series of novel developments in flow cytometry hardware, software, and dye chemistry, the use of these advances has been severely limited by the lack of commercial availability of spectrally distinct directly labeled primary and secondary antibodies. Although labeled secondary antibodies directed at individual isotype-specific targeting antibodies (e.g., anti-IgG_1 isotype antibodies) exist, it is not possible to use this type of labeled antibody to detect more than one of the same isotype of an antibody (e.g., an IgG_1 isotype antibody) in a single sample due to cross-reactivity. The current invention overcomes these limitations by providing for a convenient and extremely versatile method of rapidly labeling either small or large quantities of any primary antibody including primary antibodies of the same isotype to be used in, for example, multicolor flow cytometry and on Western blots. This advance in multicolor systems has a number of advantages over current two- and three-color flow cytometric measurements. For example, no combination of one-color stains can accurately enumerate or be used to isolate $CD3^+$ $CD4^+$ $CD8^-$ T cells (excluding, for example $CD3^+$ $CD4^+$ $CD8^+$ T cells and small $CD4^+$ monocytes). The use of cell membrane markers to study leukocyte composition in blood and tissue serves as an example of an analytical monoclonal antibody application, particularly in combination with flow cytometry. It is also the example most relevant to studies of the immune system, because the cellular composition of blood and

lymphoid tissue provides a 'window', allowing the analysis and monitoring of the immune system.

The methods of the invention can also be used in immunofluorescence histochemistry. This technique involves the use of antibodies labeled with fluorophores to detect substances within a specimen. The pathologist derives a great deal of information of diagnostic value by examining thin sections of tissue in the microscope. Tissue pathology is particularly relevant to, for example, the early diagnosis of cancer or premalignant states, and to the assessment of immunologically mediated disorders, including inflammation and transplant rejection. The problems associated with immunofluorescence histochemistry, however, stem from the limitations of the methods currently available for use in such application. For example, directly labeling an antibody can result in antibody inactivation and requires a relatively large of amount of antibody and time to do the conjugation. It is also expensive and impractical to prepare directly labeled antibodies having variable degrees of label substitution. Similarly, indirect labeling of an antibody has problems, such as lack of secondary antibody specificity, and reliance upon primary antibody differences, including antibody isotypes and available fluorophores, to do multicolor labeling. Secondary antibody labeling is not practical where the primary antibody is from the same species or of the same isotypes. Combinations of fluorophores or other detectable labels on the same target-binding antibody, which can be readily prepared in multiple mixtures by the methods on this invention, greatly increase the number of distinguishable signals in multicolor protocols. Lack of secondary antibody specificity arises when the specimen containing the targeted moiety and target-binding antibody are from homologous species. For example, BrdU-labeled DNA in rodent tissue is detected by immunohistochemical staining. The target-binding antibody is conventionally mouse anti-BrdU, and the detecting antibody system uses an anti-mouse immunoglobulin antibody, labeled with fluorescein. Because there is homology between mouse immunoglobulin and immunoglobulins from a number of rodent species (for example, rats, mice, hamsters, etc.), the detecting antibody not only binds to the target-binding antibody, but also nonspecifically binds to immunoglobulin in the tissue. The current invention eliminates this problem by pre-forming the immunolabeling complex and allows for a simple, rapid and convenient method to proceed with labeling with two, three or more fluorescent antibodies in one experiment. Very significantly, it can always be used with primary antibodies of either the same or different isotype, and always on tissue of the same or similar species as the primary antibody.

The instant invention also has application in the field of microarrays. Microarray technology is a powerful platform for biological exploration (Schena (Ed.), Microarray Biochip

Technology, (2000)). Many current applications of arrays, also known as "biochips," can be used in functional genomics as scientists seek characteristic patterns of gene expression in different physiopathological states or tissues. A common method used in gene and protein microarray technology involves the use of biotin, digoxigenin (DIG), or dinitrophenyl (DNP) as an epitope or a "tag" such as an oligohistidine, glutathione transferase, hemagglutinin (HA), or c-myc. In this case a detectably labeled anti-biotin, anti-DIG, anti-DNP, anti-oligohistidine, anti–glutathione transferase, anti-HA, or anti–c-myc is used as the detection reagent. The instant invention allows for the use of multiple fluorophore- or enzyme-labeled antibodies, thereby greatly expanding the detection modalities and also providing for enhanced multiplexing and two-dimensional analysis capabilities.

Similarly, the invention can be used with protein microarrays and on Western blots. Protein microarrays can provide a practical means to characterize patterns of variation in hundreds of thousands of different proteins in clinical or research applications. Antibody arrays have been successfully employed that used a set of 115 antibody/antigen pairs for detection and quantitation of multiple proteins in complex mixtures (Haab et al., Genome Biology, 2, 4.1 (2001)). However, protein microarrays use very low sample volumes, which historically have significantly limited the use of antibody technology for this application. The invention of the application readily overcomes this limitation and provides a means to label antibodies with the fluorescent dyes using a very low sample volume and to automate formation of the staining complex and the staining process.

The present invention also provides a means for the specific detection, monitoring, and/or treatment of disease and contemplates the use of immunolabeling complexes to detect the presence of particular targets *in vitro*. In such immunoassays, the sample may be utilized in liquid phase, in a gel, or bound to a solid-phase carrier, such as an array of fluorophore-labeled microspheres (e.g., U.S. Pat. No. 5,981,180 and 5,736,330). For example, a sample can be attached to a polymer, such as aminodextran, in order to link the sample to an insoluble support such as a polymer-coated bead, plate, or tube. For instance, but not as a limitation, using the methods of the present invention in an *in vitro* assay, antibodies that specifically recognize an antigen of a particular disease are used to determine the presence and amounts of this antigen.

Likewise, the immunolabeling complexes of the present invention can be used to detect the presence of a particular target in tissue sections prepared from a histological specimen. Preferably, the tissue to be assayed will be obtained by surgical procedures, e.g., biopsy.

The excised tissue will be assayed by procedures generally known in the art, e.g. immunohistochemistry, for the presence of a desired target that is recognized by an immunolabeling complex, as described above. The tissue may be fixed or frozen to permit histological sectioning. The immunolabeling complex may be labeled, for example with a dye or fluorescent label, chemical, heavy metal or radioactive marker to permit the detection and localization of the target-binding antibody in the assayed tissue. *In situ* detection can be accomplished by applying a detectable immunolabeling complex to the tissue sections. *In situ* detection can be used to determine the presence of a particular target and to determine the distribution of the target in the examined tissue. General techniques of *in situ* detection are well known to those of ordinary skill. See, for example, Ponder, "Cell Marking Techniques and Their Application," in MAMMALIAN DEVELOPMENT: A PRACTICAL APPROACH, Monk (ed.), 115 (1987).

For diagnosing and classifying disease types, tissues are probed with an immuno-labeled complex, as defined above, that comprises a target-binding antibody to a target antigen associated with the disease, e.g., by immunohistochemical methods. Where the disease antigen is present in body fluids, such immuno-labeled complexes comprising a target-binding antibody to the disease antigen are preferably used in immunoassays to detect a secreted disease antigen target.

Detection can be by a variety of methods including, for example, but not limited to, flow cytometry and diagnostic imaging. When using flow cytometry for the detection method, the use of microspheres, beads, or other particles as solid supports for antigen–antibody reactions in order to detect antigens or antibodies in serum and other body fluids is particularly attractive. Flow cytometers have the capacity to detect particle size and light scattering differences and are highly sensitive fluorescence detectors. Microfluidic devices provide a means to perform flow-based analyses on very small samples.

Alternatively, one can use diagnostic imaging. The method of diagnostic imaging with radiolabeled antibodies is well known. See, for example, Srivastava (ed.), RADIOLABELED MONOCLONAL ANTIBODIES FOR IMAGING AND THERAPY, Plenum Press (1988); Chase, "Medical Applications of Radioisotopes," in REMINGTON'S PHARMACEUTICAL SCIENCES, 18th Edition, Gennaro et al. (eds.) Mack Publishing Co., 624 (1990); and Brown, "Clinical Use of Monoclonal Antibodies," in BIOTECHNOLOGY AND PHARMACY, Pezzuto et al. (eds.), Chapman & Hall, 227 (1993). This technique, also known as immunoscintigraphy, uses a gamma camera to detect the location of gamma-emitting

radioisotopes conjugated to antibodies. Diagnostic imaging is used, in particular, to diagnose cardiovascular disease and infectious disease.

Thus, the present invention contemplates the use of immuno-labeled complexes to diagnose cardiovascular disease. For example, immuno-labeled complexes comprising anti-myosin antibodies can be used for imaging myocardial necrosis associated with acute myocardial infarction. Immuno-labeled complexes comprising antibodies that bind platelets and fibrin can be used for imaging deep-vein thrombosis. Moreover, immuno-labeled complexes comprising antibodies that bind to activated platelets can be used for imaging atherosclerotic plaque.

Immuno-labeled complexes of the present invention also can be used in the diagnosis of infectious diseases. For example, immuno-labeled complexes comprising antibodies that bind specific bacterial antigens can be used to localize abscesses. In addition, immuno-labeled complexes comprising antibodies that bind granulocytes and inflammatory leukocytes can be used to localize sites of bacterial infection. Similarly, the immuno-labeled complexes of the present invention can be used to detect signal transduction in cells, the products of signal transduction, and defects, inhibitors, and activators of signal transduction.

Numerous studies have evaluated the use of antibodies for scintigraphic detection of cancer. Investigations have covered the major types of solid tumors such as melanoma, colorectal carcinoma, ovarian carcinoma, breast carcinoma, sarcoma, and lung carcinoma. Thus, the present invention contemplates the detection of cancer using immuno-labeled complexes comprising antibodies that bind tumor markers (targets) to detect cancer. Examples of such tumor markers include carcinoembryonic antigen, α-fetoprotein, oncogene products, tumor-associated cell surface antigens, and necrosis-associated intracellular antigens. In addition to diagnosis, antibody imaging can be used to monitor therapeutic responses, detect recurrences of a disease, and guide subsequent clinical decisions and surgical procedures. *In vivo* diagnostic imaging using fluorescent complexes that absorb and emit light in the near infrared (such as those of the Alexa Fluor 700 and Alexa Fluor 750 dyes) is also known.

EXAMPLES

The following examples describe specific aspects of the invention to illustrate the invention and to provide a description of the methods for those of skill in the art. The examples should

not be construed as limiting the invention, as the examples merely provide specific methodology useful in understanding and practicing the invention.

Exampl 1. Preparation of Fc antigen

Purified mouse and rabbit IgG was fragmented with the proteolytic enzyme papain (CURRENT PROTOCOLS IN CELL BIOLOGY, 16.4.1–16.4.10 (2000)). A 12 mL solution of mouse IgG was prepared at ~2 mg/mL in phosphate-buffered saline (PBS). A solution containing 0.1 mg of papain in digestion buffer (PBS, 0.02 M EDTA, 0.02 M cysteine) was added to the antibody and allowed to react at 37°C for 16 hours. The digestion was terminated by the addition 20 µL of 0.3 M iodoacetamide in PBS. The fragments were dialyzed against 2 L of PBS for 16 hours at 4°C. The Fc fragment was purified on a protein G–Sepharose CL-4B column. The bound fraction containing the Fc fragment was eluted from the column using 50–100 mM glycine/HCl buffer, pH 2.5–2.8. The eluate was collected in 1 mL fractions. The pH of the protein fractions was immediately raised to neutral by addition of 100 µL of either 500 mM phosphate or Tris buffer, pH 7.6, to each 1 mL fraction. The solution was then loaded onto a Sephacryl S-200 Superfine size-exclusion column and fractions corresponding to a molecular weight of ~50 kDa were collected and analyzed by SDS-PAGE and HPLC.

Example 2. Production of Anti–Fc antibodies.

Polyclonal antibodies specific for the Fc region of an antibody were raised in goats against the purified FC region of an antibody from a different species (Example 1). Methods of immunizing animals are well known in the art, and suitable immunization protocols and immunogen concentrations can be readily determined by those skilled in the art (Current Protocols in Immunology 2.4.1-9 (1995); ILAR Journal 37, 93 (1995)). Briefly, individual goats were immunized with purified mouse Fc or purified rabbit Fc fragments. The initial immunization in 50% Freund's complete adjuvant (1000 µg conjugate (half subcutaneous, half intramuscularly)) was followed by 500 µg conjugate per goat in Freund's incomplete adjuvant two and four weeks later and at monthly intervals thereafter. Antibodies were purified from serum using protein A–Sepharose chromatography. Antibodies against mouse Fc isotypes can be prepared by starting with isotype-selected mouse Fc antigens. Rabbits have a single Fc isotype. Characterization of the selectivity and cross-reactivity of isotype-specific antibodies is by standard techniques, including HPLC.

Exampl 3. Preparation of Fab fragments.

Fragmentation of the goat anti–(mouse Fc) antibody to the monovalent Fab fragment was carried out using the proteolytic enzyme, papain, as described in Example 1. Following dialysis against PBS, the Fab fragment was purified on a protein A–Sepharose CL-4B column. The unbound fraction containing the Fab fragment and the papain was collected. This solution was then loaded onto a Sephacryl S-200 Superfine size-exclusion column and fractions corresponding to a molecular weight of ~50 kDa were collected and analyzed by SDS-PAGE. The Fab fragments of goat anti–(rabbit Fc) can be prepared similarly.

Example 4. Preparation of the labeled antibody immunoglobulin-binding protein or the non-antibody immunoglobulin-binding peptide and protein conjugates in homogeneous solution.

Conjugates of antibody immunoglobulin-binding protein or the non-antibody immunoglobulin-binding peptides or proteins with low molecular weight dyes and haptens such as biotin or digoxigenin are typically prepared from succinimidyl esters of the dye or hapten, although reactive dyes and haptens having other protein-reactive functional groups are also suitable. The typical method for protein conjugation with succinimidyl esters is as follows. Variations in molar ratios of dye-to-protein, protein concentration, time, temperature, buffer composition and other variables that are well known in the art are possible that still yield useful conjugates.

A protein solution of the Fab fragment of goat anti-(rabbit Fc), goat anti–(mouse Fc), protein A, protein G, or protein L or an immunoglobulin-binding peptide (e.g., a peptide identified by screening a library of peptides) is prepared at ~10 mg/mL in 0.1 M sodium bicarbonate (pH ~8.3). The labeling reagents are dissolved in a suitable solvent such as DMF at ~10 mg/mL. Predetermined amounts of the labeling reagents are added to the protein solution with stirring. A molar ratio of 10 moles of dye to 1 mole of protein is typical, though the optimal amount can be varied with the particular labeling reagent, the protein being labeled and the protein's concentration. The optimal ratio was determined empirically. When optimizing the fluorescence yield and determining the effect of degree of substitution (DOS) on the conjugate's brightness, it is typical to vary the ratio of reactive dye to protein over a several-fold range. The reaction mixture is incubated at room temperature for a period that is typically one hour or on ice for several hours. The dye–protein conjugate is typically separated from unreacted reagents by size-exclusion chromatography, such as on BIO-RAD P-30 resin equilibrated with PBS. The initial, protein-containing band is collected and the DOS is determined from the absorbance at the absorbance maximum of each fluorophore, using the extinction coefficient of the free fluorophore. The DOS of nonchromophoric labels,

such as biotin, is determined as described in Haugland (Haugland et al., Meth. Mol. Biol. 45, 205 (1995); Haugland, Meth. Mol. Biol. 45, 223 (1995); Haugland, Meth. Mol. Biol. 45, 235 (1995); Haugland, Current Protocols in Cell Biol. 16.5.1–16.5.22 (2000)). Using the above procedures, conjugates of goat anti–(mouse Fc) and goat anti–(rabbit Fc) were prepared with several different Alexa Fluor dyes, with Oregon Green dyes, with biotin-X succinimidyl ester, with desthiobiotin-X succinimidyl ester, with succinimidyl 3-(2-pyridyldithio)propionate (SPDP) and with succinimidyl trans-4-(maleimidylmethyl)cyclohexane-1-carboxylate (SMCC).

Some dye conjugates of protein A and protein G, including those of some Alexa Fluor dyes, are commercially available, such as from Molecular Probes. Inc. (Eugene, OR). The interspecies specificity and approximate affinity of some other non-antibody immunoglobulin-binding proteins bind to segments of a target antibody, such as that of protein A and protein G are known (Langone, Adv. Immunol. 32, 157 (1982); Surolia et al., Trends Biochem. Sci. 7, 74 (1982); Notani et al., J. Histochem. Cytochem. 27, 1438 (1979); Goding, J. Immunol. Meth. 20, 241 (1978); J. Immunol. Meth. 127, 215 (1990); Bjorck et al., J. Immunol. 133, 969 (1984)).

In addition, labeling reagents (goat Fab anti–(mouse Fc), goat Fab anti–(mouse lambda light chain), goat Fab anti–(mouse kappa light chain), protein A, protein G, protein L, lectins, single-chain fragment variable antibodies (ScFv)) conjugated to the detectable labels of R-phycoerythrin (R-PE), allophycocyanin (APC), tandem conjugates of phycobiliproteins with chemical dyes including several Alexa Fluor dyes, horseradish peroxidase (HRP), *Coprinus cinereus* peroxidase, *Arthromyces ramosus* peroxidase, glucose oxidase and alkaline phosphatase (AP) were or can be prepared by standard means (Haugland et al., Meth. Mol. Biol. 45, 205 (1995); Haugland, Meth. Mol. Biol. 45, 223 (1995); Haugland, Meth. Mol. Biol. 45, 235 (1995); Haugland, Current Protocols in Cell Biol 16.5.1–16.5.22 (2000)). Fusion proteins, such as of protein G or protein A with detectable labels such as luciferin, aequorin, green-fluorescent protein and alkaline phosphatase are also known that are suitable for practice of the invention (Sun et al., J. Immunol. Meth. 152, 43 (1992); Eliasson et al., J. Biol. Chem. 263, 4323 (1988); Eliasson et al., J. Immunol. 142, 575 (1989)).

Immunoglobulin heavy and light chains, like most secreted and membrane bound proteins, are synthesized on membrane-bound ribosomes in the rough endoplasmic endoplasmic reticulum where N-linked glycosylation occurs. The specificity of lectins for carbohydrates, including N-linked glycoproteins, is also known (EY laboratories, Inc. Lectin Conjugates Catalog, 1998).

Example 5. Preparation of the labeled antibody immunoglobulin-binding protein or the non-antibody immunoglobulin-binding peptide and protein conjugates while bound to an affinity matrix.

Unlabeled Fab fragment for goat anti–(mouse Fc) (prepared as in Example 3) was bound to agarose-immobilized mouse IgG for one hour. Following a wash step with bicarbonate buffer, pH 8.3, the complex of immobilized IgG and unlabeled Fab was labeled for one hour at room temperature with the succinimidyl ester of the amine-reactive label. Unconjugated dye was eluted with bicarbonate buffer, and then the covalently labeled Fab fragment was eluted with 50–100 mM glycine/HCl buffer, pH 2.5–2.8. The eluate was collected in 1 mL fractions. The pH of the protein fractions was immediately raised to neutral by addition of 100 µL of either 500 mM phosphate or Tris buffer, pH 7.6, to each 1 mL fraction. Variations of the reagent concentrations, labeling times, buffer composition, elution methods and other variables are possible that can yield equivalent results. Conjugates of the Fab fragment of goat anti–(rabbit Fc) and of protein G and protein A are prepared similarly.

Example 6. Comparison of the Alexa Fluor 488 dye–labeled Fab fragments of goat anti–(mouse Fc) prepared as in Example 4 and as in Example 5.

Conjugates of the Fab fragment of goat anti–(mouse Fc) with the Alexa Fluor 488 succinimidyl ester were separately prepared, as described in Examples 4 and 5. The conjugates had estimated degrees of substitution of ~1.9 (labeled as in Example 4) and ~3.0 (labeled as in Example 5), respectively, and virtually identical absorption and emission spectral maxima. When excited at 488 nm, conjugates prepared using the fragment prepared as described in Example 5 were about 3.2-times more fluorescent than using the fragments that were prepared in **Example 4** (Figure 8) as detected by flow cytometry when bound to CD3 on Jurkat T cells. Similar results were observed with other dyes.

Example 7. Preparation of a labeling reagent from protein G and albumins.

Native protein G has a high affinity binding (nanomolar) site for albumins, in particular ovalbumin. Equal weights of protein G and Texas Red ovalbumin (Molecular Probes. Inc.) were dissolved in PBS, pH 7.5. After one hour, the resulting complex was separated on a Sephacryl S-200 Superfine size-exclusion column and analyzed by SDS-PAGE and HPLC. Alternatively, the protein G is combined with a labeled albumin while the protein G is immobilized on any of the several immunoglobulins to which it binds, and the excess labeled

albumin is washed away preceding elution of the albumin-labeled protein G complex from the matrix.

Exampl 8. Preparation of an immunolabeling complex on a very small scale.

Submicrogram quantities of a target-binding antibody were complexed with submicrograms of a labeling reagent in varying molar ratios of between about 1:1 and 1:20 to prepare an immunolabeling complex that was suitable for staining a sample. For instance, 0.1 µg of mouse monoclonal anti–α-tubulin in 1 µL PBS with 0.1% BSA was complexed with 0.5 µg of the Alexa Fluor 488 dye–labeled Fab fragment of goat anti–(mouse Fc) (prepared as in Example 4) or with 0.1 µg of the Alexa Fluor 488 dye–labeled Fab fragment of goat anti–(mouse Fc) (prepared as in Example 5) in 5 µL of PBS for 10 minutes at room temperature. The immunolabeling complex can be used immediately for staining tubulin in fixed-cell preparations (Example 16) or any excess unbound Alexa Fluor 488 dye–labeled Fab fragment of goat anti–(mouse Fc) in the immunolabeling complex can be captured with non-immune mouse IgG (Example 9) for combination with other antibody conjugates, including those of targeting antibodies that have been directly conjugated to other labels. Rabbit antibodies were labeled similarly using labeled goat anti–(rabbit Fc). Labeling of targeting antibodies with a labeled protein A, protein L, protein G, protein G complexed with a labeled albumin, or other immunoglobulin-binding peptides or proteins proceeds similarly. In the case of a mouse (or rat) monoclonal antibody, it is preferred to use a labeled protein that is selective for the specific isotype of the primary antibody (e.g. anti–(mouse IgG_1) for a mouse IgG_1 isotype primary antibody). Although some cross-reactivity for other mouse (or rat) isotypes was observed using a goat antibody that was selective for mouse IgG_1 isotype monoclonal antibodies, routine and optimal use for labeling unmatched mouse isotypes required greater amounts of immunolabeling complexes and was somewhat less reliable.

Example 9. Capturing excess immunoglobulin-binding protein by a capturing component.

Immunolabeling complexes were prepared as described in Example 8. To the immunolabeling complex was added to each tube 25 µL of a 14.1 mg/mL stock solution of unlabeled mouse IgG to capture excess immunolabeling complexes. As shown in Figure 1, not all of the immunoglobulin-binding protein was necessarily complexed with the target-binding antibody to form an immunolabeling complex. Consequently, particularly for applications in which labeling complexes of multiple primary antibodies from the same species (e.g. mouse monoclonal antibodies) or crossreacting species (e.g. mouse and human antibodies, Figure 2, Table 1) were to be used simultaneously or sequentially, it is

necessary to quench or otherwise remove any excess immunoglobulin-binding protein by use of a capturing component or by other means to avoid inappropriate labeling of the sample. The most effective capturing component to capture excess immunoglobulin-binding protein is one that contains the binding site of the targeting agent. For instance, whole mouse IgG or mouse serum was shown to be an effective and inexpensive reagent when the immunoglobulin-binding protein was bound to a segment of a mouse monoclonal antibody. The mouse IgG was added in excess to the amount of immunoglobulin-binding protein and incubated for a period of approximately 1–5 minutes, or longer.

It is preferred to prepare the immunolabeling complex and then add the capturing component shortly before the experiment. The rapid quenching effect permits this to be done within minutes of performing labeling of the sample by the immunolabeling complex. If desired, the excess capturing component can be removed following labeling of the sample by a simple wash step. Alternatively, fixation of the stained sample by aldehyde-based fixatives or other reagents or methods subsequent to incubation with the immunolabeling complex can provide permanent immobilization of the immunolabeling complex on its target in the sample. As an alternative to adding a soluble capturing component to the immunolabeling complex, the capturing component can be immobilized on an insoluble matrix such as agarose and the immunolabeling complex contacted with that matrix. A preferred matrix when labeling mouse antibodies to mouse antigens is mouse IgG immobilized on agarose. Excess labeled anti–rabbit antibodies can be captured using rabbit IgG that is free in solution or immobilized. Alternatively, the immunolabeling complex can be separated from any capturing component by chromatographic or electrophoretic means.

Example 10. HPLC analysis of a labeling complex.

In order to analyze the success and extent of complex formation of the labeling reagent with the target-binding antibody, size exclusion HPLC of the samples was performed. For instance, a complex of Alexa Fluor 488 dye–labeled goat Fab anti–(mouse Fc) with a monoclonal mouse anti–tubulin in molar ratios of approximately 1:1, 3:1, 5:1 and 10:1. These were separated by analytical HPLC using a BioSep S-3000 column and eluting with 0.1 M NaP_i, 0.1 M NaCl, pH 6.8, at a flow rate of 0.25 mLs/min. An example of the separation using the 5:1 molar ratio (Figure 6) demonstrates that, using this molar ratio, formation of the labeled complex is essentially quantitative.

Example 11. Cross-reactivity of goat Fab anti–(mouse Fc) to other species of IgG.

Microplates were equilibrated overnight with IgG from a mouse or non-mouse species, and then further blocked with BSA. Variable amounts of the biotinylated Fab fragment of goat anti–(mouse Fc) were added to each well and allowed to bind. After washing, streptavidin–HRP and the Amplex Red peroxidase substrate were added. HRP activity was detected by the addition of H_2O_2 using the Amplex Red Peroxidase Assay Kit (Molecular Probes, Inc., Eugene, OR). Reactions containing 200 µM Amplex Red reagent, 1 U/mL HRP and 1 mM H_2O_2 (3% solution) in 50 mM sodium phosphate buffer, pH 7.4, were incubated for 30 minutes at room temperature. Fluorescence was measured with a fluorescence microplate reader using excitation at 560 ± 10 nm and fluorescence detection at 590 ± 10 nm. Background fluorescence, determined for a no-H_2O_2 control reaction, was subtracted from each value (Table 1 and Figure 2). Table 1 shows that the goat anti–(mouse Fc) antibody because of the highly conserved structure of the Fc region of an antibody it can be used to complex other non-mouse antibodies, including rat, and human antibodies. The goat anti–mouse IgG antibody reaction with mouse antibody was set at 100% and the crossreacting antibodies were expressed as a percentage compared the mouse on mouse data. The data in Table 1 show that the Fab fragment of the goat anti–(mouse Fc) antibody of the current invention does not strongly bind to the goat or sheep Fc domain; however, one skilled in the art could generate antibodies that will react with the goat and sheep Fc domain or the Fc domain of any other species. Biotinylated Fab goat anti–(mouse Fc) was used in this example because it provided a convenient method to quantitate the amount of crossreactivity in a conventional method but it could have been accomplished using a fluorophore Fab labeled goat anti–(mouse Fc). It was demonstrated by HPLC (as in Example 10) that Alexa Fluor 488 dye–labeled goat anti–(rabbit Fc) bound to rabbit primary antibodies.

Table 1. Cross-reactivity of goat anti–mouse IgG antibody with other non-mouse antibodies.

Species	Crossreactivity	% Fluorescence
Mouse	++++	100
Rat	+++	80.7
Human	++	66.7
Rabbit	+	16.9
Goat	-	6.5
Sheep	-	5.7

Exampl 12. Determination of the optimal molar ratio of immunoglobulin-binding protein to target antibody using a microplate assay.

To 1.6 µg of mouse monoclonal anti-biotin (MW ~145,000) in 8.0 µL PBS was added varying amounts of the Alexa Fluor 488 dye–labeled Fab fragment of goat anti–(mouse Fc) (MW ~50,000) (prepared as in **Exampl 4**) to form an immunolabeling complex. After equilibration for 20 min, a 100 µL aliquot was added to a 96-well microplate coated with biotinylated BSA. After 30 minutes, the plates were washed and the residual fluorescence was quantitated using a fluorescence microplate reader using excitation at 485 +/- 10 nm and detecting emission at 530 +/- 12.5 nm. As shown in Figure 3, a molar ratio of the Alexa Fluor 488 dye–labeled Fab fragment of goat anti–(mouse Fc) to the anti-biotin between 5 to 20 was sufficient to form appreciably detectable complexes (Figure 3; fluorescence quantitated, performed in triplicate (circles); control experiments performed but without adding the primary anti-biotin antibody (solid squares)). A molar ratio of about 5 to about 10 was preferred for this pair of immunoglobulin-binding protein and target antibody. This ratio can be varied somewhat to increase or decrease the signal or to affect the consumption of valuable reagents. The weight ratio of immunoglobulin-binding protein to target-binding antibody is particularly affected by the actual molecular weight of the immunoglobulin-binding protein.

For instance, equal weights of the dye-labeled goat Fab anti–(mouse Fc) (prepared as in Example 5) and an intact mouse primary antibody, which corresponds to an approximately 3 to 1 molar ratio, usually yields suitable labeling complexes. Fluorescence intensity (or enzymatic activity) of the immunolabeling complex is readily adjusted by a corresponding adjustment of the amount of labeled Fab fragment used.

Similar analyses of the ratio for other labeling reagents (including those of labeled protein A, protein G, protein L, IgG-binding peptides and antibodies to other segments of the primary antibody), and for conjugates of labels other than Alexa Fluor 488 dye (including enzymes in combination with the appropriate enzyme substrates) are done essentially as described in this example.

Example 13. Dissociation rate of the immunolabeling complex.

A pre-equilibrated immunolabeling complex was prepared from 50 µg of an Alexa Fluor 488 dye–labeled Fab fragment of goat anti–(mouse Fc) and 15 µg of an anti-biotin monoclonal antibody (mAb). The immunolabeling complex was rapidly diluted with capturing component sufficient to give a 6.2 molar excess over the anti-biotin mAb. At various times, an aliquot was taken and added to a microplate well containing an excess of biotinylated BSA. After 30

minutes, the plates were washed and the remaining fluorescence was quantitated. Displacement of the labeling reagent from the target-binding antibody through exchange was measured by any time-dependent decrease in fluorescence in the microplate well. For example the fragments prepared as described in Example 4 had 68 percent fragments bound to the target-binding antibody after 30 minutes compared to 87 percent of bound fragments that were prepared according to Example 5. One hour showed a similar decrease, 56 percent and 68 percent respectively. The labeling reagent was shown to undergo a stable interaction with the target-binding antibody, with a lifetime for half exchange under these conditions of 3.5 hours. Dissociation rates were measured for labeling reagent prepared according to Example 4 and for labeling reagent prepared according to Example 5, demonstrating the greater stability of immunolabeling complexes made using the labeling reagents prepared according to Example 5.

Example 14. Protocol for staining cultured cells with a single immunolabeling complex.

Culturable cells, such as bovine pulmonary artery endothelial cells (BPAEC), were grown on a 22 x 22 mm glass coverslip. The cells were fixed for 10 minutes using 3.7% formaldehyde in DMEM with fetal calf serum (FCS) at 37°C. The fixed cells were washed 3 times with PBS. The cells were permeabilized for 10 min with 0.02% Triton X-100 in PBS, washed 3X with PBS and blocked for 30 min with 1% BSA in PBS. Variations of the cell type and cell preparation, fixation, and permeabilization methods, including methods for antigen retrieval, are well known to scientists familiar with the art. An immunolabeling complex was prepared as described in Example 8. The immunolabeling complex was added directly to the fixed and permeabilized cells in an amount sufficient to give a detectable signal if there is a binding site for the primary antibody present in the sample. After an incubation period that was typically 10–60 minutes (usually about 15–30 minutes), the cells were washed with fresh medium and the labeling was evaluated by methods suitable for detection of the label. Staining by the immunolabeling complex can be additionally preceded, followed by or combined with staining by additional reagents, such as DAPI, which yields blue-fluorescent nuclei.

Example 15. Protocol for staining cultured cells with multiple immunolabeling complexes.

Cells were fixed and permeabilized as described in Example 14. Multiple immunolabeling complexes were individually prepared from a variety of labeling reagents, according to the procedure described in Example 8. The multiple immunolabeling complexes were either used individually or sequentially to stain the cells, according to the procedure described in

Exampl 14, or two or more immunolabeling complexes were formed then co-mixed in a single staining solution and used to simultaneously stain the sample. The optimal method for cell fixation and permeabilization and the best ratio for combination of the immunolabeling complexes are typically determined by preliminary experimentation using single immunolabeling complexes or multiple immunolabeling complexes used in combination. A first immunolabeling complex was prepared from an Alexa Fluor 488 dye–labeled Fab fragment of goat anti–(mouse Fc) and mouse monoclonal anti–α-tubulin, a second immunolabeling complex was prepared from an Alexa Fluor 568 dye–labeled Fab fragment of goat anti–(mouse Fc) and mouse monoclonal anti-vimentin (anti-vimentin was an ascites fluid preparation) and a third immunolabeling complex was prepared from an Alexa Fluor 647 dye–labeled Fab fragment of goat anti–(mouse Fc) and mouse monoclonal anti-cdc6 peptide antibody (Molecular Probes). Aliquots of the three different immunolabeling complexes were combined and used to stain BPAE cells for 30 minutes, washed with fresh medium and observed by fluorescence microscopy using optical filters appropriate for the three dyes. In this example, some cells showed cytoplasmic staining by the anti-vimentin antibody, nuclear staining by the anti-cdc6 peptide antibody and staining of mitotic spindles by the anti–α-tubulin antibody, indicative of a cell in mitosis. Staining by the immunolabeling complexes was additionally preceded, followed by or combined with staining by additional reagents, such as Alexa Fluor 350 phalloidin, which yielded blue-fluorescent actin filaments in the above example.

The immunolabeling complexes that are used in combination do not have to be targeted toward antibodies from the same species. For instance, complexes of Alexa Fluor 488 dye–labeled goat anti–(mouse IgG_1 Fc) with a mouse IgG_1 monoclonal target-binding antibody and an Alexa Fluor 594 dye–labeled goat anti–(rabbit Fc) with a rabbit primary target-binding antibody can be prepared and used in combined staining protocols.

Example 16. Protocol for staining tissue with a single immunolabeling complex.

A mouse intestine cryosection (University of Oregon histology core facility), a cross-section of about 16 μm thickness, was mounted on a slide. The intestine was perfused and fixed with 4% formaldehyde prior to dissection, embedding, and sectioning. The tissue section was rehydrated for 20 minutes in PBS. An immunolabeling complex was prepared as described in Example 8. Briefly, 0.1 μg of mouse monoclonal anti–cdc6 peptide (a nuclear antigen) in 1 μL PBS with 0.1% BSA was complexed with 0.5 μg of the Alexa Fluor 350 dye–labeled Fab fragment of goat anti–(mouse IgG_1 Fc) (prepared as in Example 4) in 5 μL of PBS for 10 minutes at room temperature. Excess Fab fragment of goat anti–(mouse IgG_1

Fc) was captured with 25 µL of a 14.1 mg/mL stock of unlabeled mouse IgG. The tissue was permeabilized with 0.1% Triton X-100 for 10 min. The tissue was washed two times with PBS and was blocked in 1% BSA for 30 min. The immunolabeling complex was added directly to the tissue for 30 minutes and washed three times in PBS. The sample was mounted in Molecular Probes' Prolong antifade mounting medium and observed by fluorescence microscopy using optical filters appropriate for the Alexa Fluor 350 dye. Results showed that the mouse monoclonal anti–cdc6 peptide immunolabeling complex showed specific nuclear labeling in the mouse intestine tissue section. Variations of the tissue type and tissue preparation, fixation and permeabilization methods, mounting methods, including methods for antigen retrieval, are well known to scientists familiar with the art.

Example 17. Staining of a tissue target in combination with tyramide signal amplification (TSA).

Mouse brain cryosections were labeled with a pre-formed complex of horseradish peroxidase (HRP)–labeled goat anti–(mouse IgG_1 Fc) antibody and a mouse IgG_1 monoclonal anti–(glial fibrillary acidic protein (GFAP)) prepared essentially as in Example 8 using a molar ratio of labeling reagent to monoclonal antibody of 3. Staining of the mouse tissues was essentially as in Example 16. The staining localization and intensity was compared to that of (a) goat anti–mouse IgG HRP conjugate and mouse anti-GFAP, (b) the Alexa Fluor 488 dye–labeled Fab fragment of goat anti–(mouse IgG_1 Fc) antibody complex of mouse anti-GFAP, (c) Alexa Fluor 488 goat anti–mouse IgG secondary antibody and mouse anti-GFAP, and (d) a direct conjugate of the Alexa Fluor 488 dye with mouse anti-GFAP. The HRP-conjugated probes were incubated with Alexa Fluor 488 tyramide using TSA Kit #2 (Molecular Probes, Inc.) according to standard procedures. The tissue staining patterns in each case were similar and consistent with the expected staining pattern of mouse anti-GFAP and staining was essentially free of nonspecific background. The relative fluorescence intensities of staining measured by digital imaging were sequentially: 541 relative intensity units for the HRP–goat anti–(mouse IgG_1 Fc) complex of mouse anti-GFAP and (using the combinations indicated by the letters above): (a) 539, (b) 234, (c) 294, and (d) 255 relative intensity units.

Example 18. Staining of live cells by multiple immunolabeling complexes.

A first immunolabeling complex was prepared from an Alexa Fluor 488 dye–labeled Fab fragment of goat anti–(mouse IgG_1 Fc) and mouse monoclonal anti–(human CD8), a second

immunolabeling complex was prepared from an R-phycoerythrin–conjugated Fab fragment of goat anti–(mouse IgG_1 Fc) and mouse anti–(human CD3), and a third immunolabeling complex was prepared from an Alexa Fluor 647 dye–labeled Fab fragment of goat anti–(mouse IgG_1 Fc) and mouse anti–(human CD4). The complexes were prepared as described in Example 8 and were each blocked with 20 µg (1.3 µL of 14.1 µg/mL) of mouse IgG for 10 minutes at room temperature. The first immunolabeling complex was added to 100 µL of whole blood and incubated for 15 min. The cells were washed with PBS and 280.5 µL of the second immunolabeling complex was added and incubated for 15 min. The cells were again washed, and 46.2 µL of the third labeling complex was added and incubated for 15 min. After the final incubation, the red blood cells were lysed with cell-lysis buffer. The cells were resuspended in 1% formaldehyde/PBS and analyzed on a FACS Vantage flow cytometer using a 488 nm argon-ion laser for excitation of the first and second immunolabeling complexes and a 633 nm red He-Ne laser for excitation of the third immunolabeling complex (Figures 5a, 5b). The emission band pass filters used for selective detection of the dyes are 525 +/- 10 nm for the Alexa Fluor 488 (CD8), 585 +/-21 nm for R-PE (CD3) and 675 +/- 10 nm for the Alexa Fluor 647 dye (CD4). Figures 5a and 5b show that the instant invention can be used in a 3-color immunophenotyping experiment using peripheral blood lymphocytes. CD3-positive T cells were stained with the R-phycoerythrin–conjugated Fab fragment of goat anti–(mouse Fc) and mouse anti–(human CD3), upper left (UL) quadrant, Figure 5a. CD4-positive cells, a T cell subset, are identified using Alexa Fluor 647 dye–labeled Fab fragment of goat anti–(mouse IgG_1 Fc) and mouse anti–(human CD4), UL quadrant, Figure 5b and CD8-positive T cells, a T cell subset, were identified using Alexa Fluor 488 dye–labeled Fab fragment of goat anti–(mouse IgG_1 Fc) and mouse monoclonal anti–(human CD8), lower right (LR) quadrant, Figure 5b.

Exposed antigens of live cells, including cultured cells and cells from biological fluids such as blood and cerebrospinal fluid can be simultaneously or sequentially stained by combinations of immunolabeling complexes, including antibodies to the same target labeled with two or more separately detectable immunoglobulin-binding proteins.

Example 19: The dye–labeled Fab fragment of goat anti–(mouse Fc) can be utilized for the combinatorial labeling of primary antibodies, to generate a multitude of colored targets.

A first immuno-labeled complex was made by combining 2.5 µg Alexa Fluor 488 dye–labeled Fab fragment of goat anti–(mouse IgG_1 Fc) with 0.5 µg mouse anti–human CD3 (Caltag at 200 µg/mL), according to the procedure described in Example 4. A second immunolabeling complex was made by combining 5.0 µg Alexa Fluor 647 dye–labeled Fab fragment of goat

anti–(mouse IgG_1 Fc) with 0.5 µg mouse anti–human CD3, according to the procedure in Example 4. Each complex was separately incubated at room temperature for 5 minutes, and each complex was then separately combined with an excess of mouse IgG (14.1 mg/mL) for 5 min at room temperature to capture excess unbound dye-labeled Fab fragments. The two immunolabeling complexes were then added in different percentage combinations (see Table 2) to 100 µL of washed heparinized blood. The cells were incubated with the respective combinations of complexes for 20 min on ice. The red blood cells were then lysed with a cell-lysis buffer. The cells were resuspended in 1% formaldehyde/PBS and analyzed on a FacVantage flow cytometer using a 488 nm argon 633 HeNe laser for excitation and a 530 +/-10 nm band pass emission filter (FL1), and a 640 long pass filter (FL4). Five samples of different combined percentages (Table 2) were compared by flow cytometry, with signals being collected in FL1 and FL4. To determine the percentage of cells detected with each type of emission, the FL1 and FL4 intensities for each percentage combination were normalized by dividing the FL1 and FL4 channel intensities for such combinations by the intensities of the 100% Alexa Fluor 488 dye– and 100% Alexa Fluor 647 dye–labeled cells, respectively.

Table 2. Theoretical versus recovered dye–labeled Fab fragment of goat anti–(mouse IgG_1 Fc) combinatorial experiment.

Experimentally mixed percentage of cells labeled with Alexa Fluor 488 dye–labeled Fab fragment of goat anti–(mouse IgG_1 Fc)	Recovered percentage of measured cells labeled with Alexa Fluor 488 dye–labeled Fab fragment of goat anti–(mouse IgG_1 Fc)	Experimentally mixed percentage of cells labeled with Alexa Fluor 647 dye–labeled Fab fragment of goat anti–(mouse IgG_1 Fc)	Recovered percentage of measured cells labeled with Alexa Fluor 647 dye–labeled Fab fragment of goat anti–(mouse IgG_1 Fc)
100%	100%	0%	0%
75%	81%	25%	14%
50%	63%	50%	38%
25%	35%	75%	73%
0%	0%	100%	100%

Exampl 20: The immunolabeling complex can be used to detect antigens on a Western blot

Bovine heart mitochondria were isolated (Hanson et al., Electrophoresis 22, 950 (2001)). The isolated mitochondria were resuspended to ~10 mg/mL in 100 mM Tris-HCl, pH 7.8, 1 mM phenylmethylsulfonyl fluoride (a protease inhibitor), 2% SDS and insoluble material was removed by centrifugation for 10 minutes at 10,000 x g in a tabletop centrifuge. The protein concentration of the lysate was checked by the BCA assay (Pierce, Rockford, IL). Samples for gel electrophoresis were prepared by mixing lysate, water, and loading buffer to the appropriate concentrations (final concentration of loading buffer in samples: 58 mM Tris/HCl, 10% glycerol, 2% SDS, 0.02 mg/mL bromphenol blue, 50 mM DTT, pH 8.6). The samples were then heated to 90°C for 5 minutes before loading on the gel and separated on a 13% SDS-PAGE gel. Two-fold serial dilution of the extracts ranging from 8 µg of extract down to 0.03 µg were loaded on the SDS-PAGE gel. The proteins were transferred to PVDF membrane for 1.5 hours using a semi-dry transfer system according to manufacturer's directions (The W.E.P. Company, Concord, CA). The PVDF membrane was blocked for 1 hour in 5% milk.

Immunolabeling complexes were made with mouse monoclonal antibodies that recognize two different mitochondrial proteins. Alexa Fluor 647 dye–labeled Fab fragment of goat anti–(mouse IgG_1 Fc) (5 µL of a 1 mg/mL stock, prepared as in Example 4) was incubated with 21 µL (0.88 mg/mL) mouse anti–(CV-alpha) and Alexa Fluor 488 dye–labeled Fab fragment of goat anti–(mouse IgG_1 Fc) (5 µL of a 1 mg/mL stock, prepared as in Example 4) was incubated with 19 µL (0.88 mg/mL) mouse anti–(CIII-core2) (Molecular Probes, Eugene, OR). Following a 30 minute incubation, 25 µL of a 14.1 mg/mL stock of unlabeled mouse IgG was added to each tube. The immunolabeling complexes were then mixed together and brought up to 5 mL in 5% milk. The blot was incubated with the mixture of immunolabeling complexes for 1 hour at room temperature. The blot was washed twice for 5 seconds each with PBST (PBS with 0.1% Tween) and once with PBST for 15 minutes. The blot was air dried and imaged on an EG&G Wallac Imager with the appropriate filters. The Western blot revealed two distinct bands of the appropriate molecular weight. The Western blot also showed that no cross-labeling of the antibodies occurred and the detection limit was 125 ng.

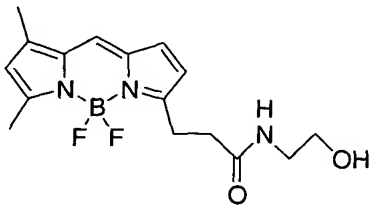
Example 21: High-throughput screening of hybridomas for identifying high affinity and high IgG producers

Microplate wells containing both a fluorescent labeled antigen of one fluorescent color label and fluorescently labeled Fab fragments of goat anti-(mouse Fc) of a different fluorescent color made by the method described in Example 4 and 5. Hybridoma supernatant is

harvested and added to the wells. If the hybridoma are producing the desired antibody, i.e. antibodies that bind to the labeled antigen, polarization of the florescence corresponding to the labeled antigen will allow visualization of those wells containing antigen specific antibody. In addition, the amount of IgG that the hybridomas produce, can be simultaneously identified by polarization of the fluorescence corresponding to the labeled Fab fragments. This method thus allows for both quantitation of the amount of antibody present in a specific amount of hybridoma supernatant and the affinity of the monoclonal antibodies for the antigen.

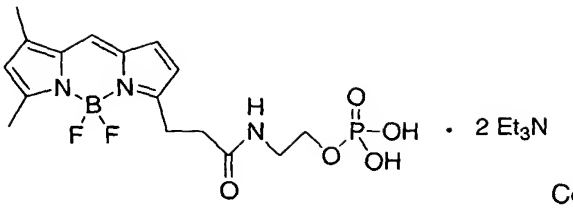
Example 22: Synthesis of phosphorylethanolamine ligand analog, Compound **2**

To an orange solution of BODIPY FL succinimidyl ester (Molecular Probes 2184, 200 mg, 0.51 mmol) in 20 mL anhydrous tetrahydrofuran was added a solution of ethanolamine (36 □L, 0.6 mmol) in 1 mL dioxane. The resulting cloudy orange mixture was stirred at room temperature for 3 hours, and concentrated in vacuo. The residue was purified by flash chromatography on silica gel using 10% methanol in chloroform as eluant to give the corresponding ethanolamine amide of BODIPY FL as 0.17 g (99%) of an orange powder: ^{1}H NMR (CD_2Cl_2) □ 7.19 (s, 1H), 6.97 (d, 1H), 6.34 (d, 1H), 6.20 (s, 1H), 6.08 (br s, 1H), 3.65 (t, 2H), 3.36 (m, 2H), 3.26 (t, 2H), 2.66 (t, 2H), 2.57 (s, 3H), 2.30 (s, 3H); LRMS m/z 335 (335 calcd for $C_{16}H_{20}N_3O_2BF_2$).



Compound **1**

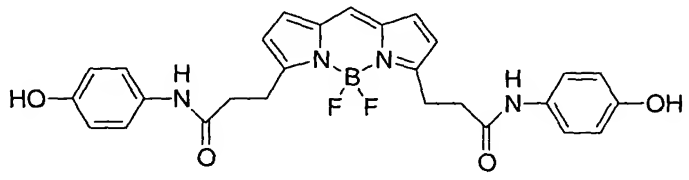
To a solution of BODIPY FL succinimidyl ester (Molecular Probes 2184, 50 mg, 0.13 mmol) in 5 mL dioxane was added a solution of O-phosphorylethanolamine (27 mg, 0.19 mmol) in 2 mL of 0.5 M triethylammonium bicarbonate. The resulting solution was kept at room temperature for 40 minutes and then concentrated to dryness. Water was twice evaporated from the residue, which was purified by chromatography on Sephadex LH-20 using water as eluant. Pure product fractions were pooled and lyophilized to give Compound **2** as an orange powder.



Compound **2**

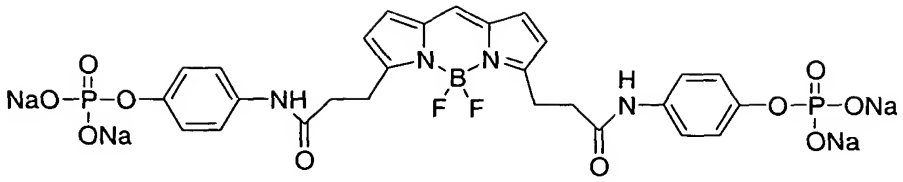
Example 23: Synthesis of phosphotyramide ligand analog, Compound **4**

To a solution of 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene-3,5-dipropionic acid (Molecular Probes 6103, 0.10 g, 0.30 mmol) in 15 mL anhydrous THF under argon was added oxalyl chloride (78 □L, 0.89 mmol) and one drop of DMF. The volatiles were removed in vacuo after 15 minutes of stirring, leaving a residue of 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene-3,5-dipropionyl chloride. This bis-acid chloride was dissolved in 15 mL anhydrous THF, and the resulting solution added dropwise to a solution of 4-aminophenol (98 mg, 0.90 mmol) and diisopropylethylamine (0.16 mL, 0.90 mmol) in 10 mL anhydrous THF with stirring. The resulting green-orange mixture was stirred at room temperature for 3h and then quenched with 10% citric acid (75 mL). The resulting mixture was extracted with ethyl acetate (2x50 mL). The extract was washed with brine (1x), dried over sodium sulfate, and concentrated to an orange residue. Flash chromatography using methanol in chloroform gave Compound **3** as an orange powder: LCMS m/z 518 (518 calcd for $C_{27}H_{25}N_4O_4BF_2$).



Compound **3**

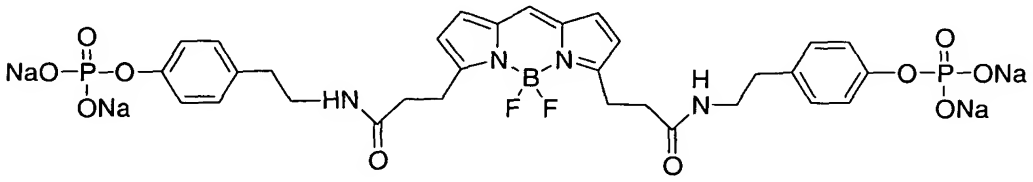
To a solution of 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene-3,5-dipropionyl chloride (0.059 mmol) in 2 mL methylene chloride was added a solution of O-phosphoryl-4-aminophenol disodium salt (34 mg, 0.15 mmol) in 5 mL DMF/0.2 mL acetic acid. The resulting mixture was stirred at room temperature for two hours and then evaporated to dryness. Toluene was evaporated from the residue, which was purified by chromatography on Sephadex LH-20 using water as eluant to give Compound **4** as an orange powder.



B596-64A-AR Compound **4**

Example 24: Synthesis of a fluorogenic phosphotyramide ligand analog, Compound **5**

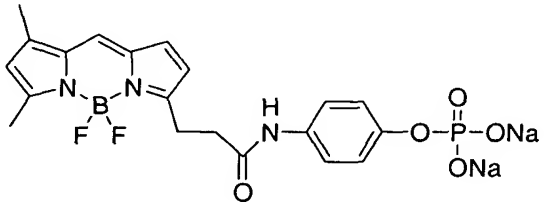
A 0.05M solution of 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene-3,5-dipropionyl chloride in anhydrous dioxane is added dropwise to a 0.1M solution of 2.5 eq O-phosphotyramine disodium salt in water (pH 8-9) with stirring. After stirring at room temperature overnight, the volatiles are removed in vacuo. The residue is purified by chromatography on Sephadex LH-20 using water as eluant to give Compound **5** as an orange powder.



Compound **5**

Example 25: Synthesis of phosphotyramide ligand analog, Compound **6**

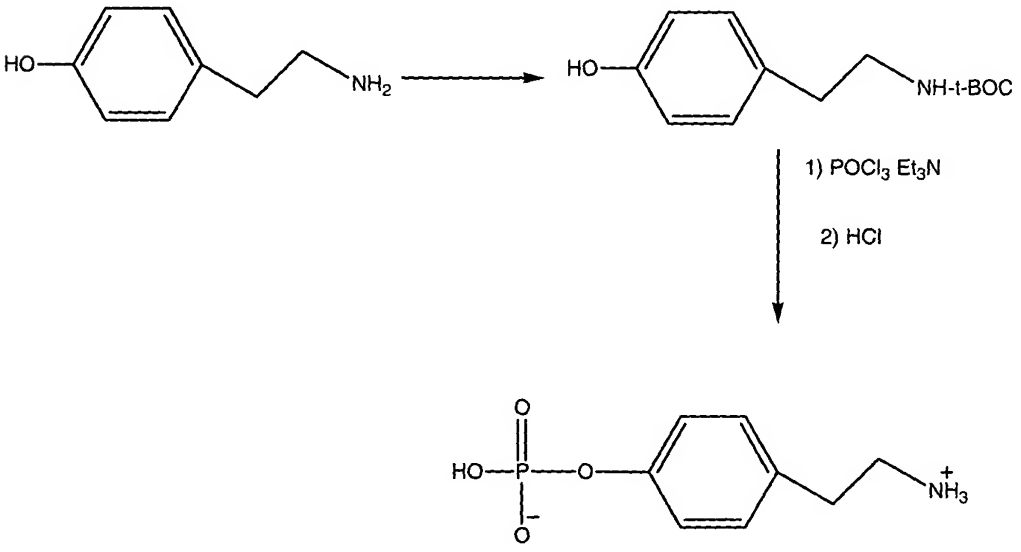
To a solution of O-phosphoryl-4-aminophenol disodium salt (0.27 mmol) in 30 mL anhydrous DMF was added diisopropylethylamine (0.23 mL, 1.3 mmol) and a solution of 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene-5,7-dimethyl-3-propionyl chloride in anhydrous dichloromethane (15 mL). The resulting mixture was stirred at room temperature overnight, then evaporated to dryness. The residue was dissolved in 1:1 methanol/water and then loaded onto a Sephadex LH-20 column, followed by gravity elution with water. Pure product fractions were combined and lyophilized to give Compound **6** as 10 mg of an orange powder.



Compound **6**

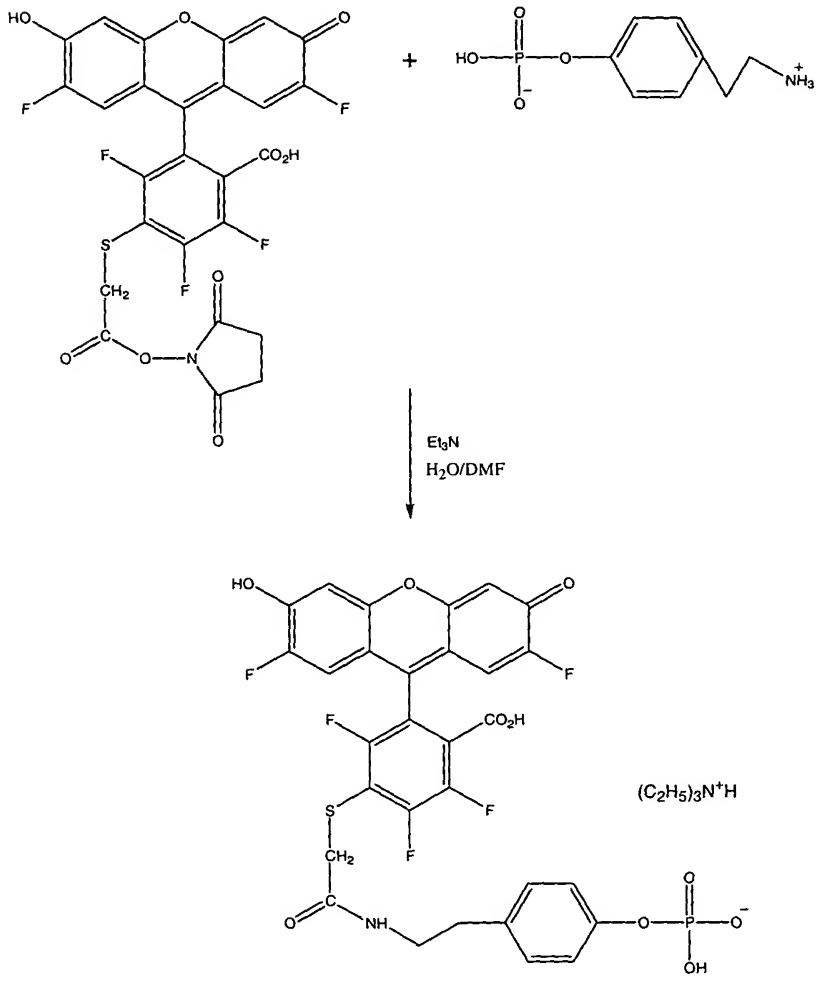
Exampl 26: Synthesis of phosphotryamide ligand analog, Compounds **7-21** and **39**

First, the intermediate O-phsphotyramine synthesized wherein the amine group was protected as t-BOC and the phosphorylation was done with $POCl_3$ and N,N-diisopropylethylamine in $CHCl_3$. The t-BOC group was removed by HCl in aqueous solution. Specifically this was accomplished wherein a suspension of tyramine (1.0 g, 7.29 mmol) in 50 ml of chloroform was added N.N-diisopropylethylamine (1.3 ml, 7.59 mmol) followed by addition of di-tert-butyl carbonate (1.60 g, 7.34 mmol) and the mixture was strirred at room temperature for 3 hours. The resulting reaction mixture was washed with 1% HCl (1x50 ml), water (2x50 ml) and then separated. Organic layer is dried over Na_2SO_4, filtered and concentrated under vacuum to give N-t-BOC protected intermediate. This intermediate (1.50 g, 6.33 mmol) is dissolved in 50 ml of chloroform and was added N,N-diisopropyl-ethylamine (1.09 ml, 6.36 mmol) and phosphorus oxychloride (580 µl, 6.34 mmol). After stirring at room temperature for 2 hours, all the chloroform was removed under vacuum and H_20 (10 ml) was added, stirred at room temperature for 2 hours. The resulting aqueous solution was subjected to 2H-2O column by elution with water. From the combined desired fractions, O-Phosphotyramine (0.52 g) is obtained. (R*f*= 0.40 (silica gel, 20% water is acetonitrile)).



Next the succinimidyl ester version of the dye to O-Phosphotyramine was added to give Compound **7**. This is accomplished wherein a solution of O-Phosphotyramine (3 mg, 0.01 mmol) and triethylamine (5µl, 0.04 mmol) in 500 µl of water is added a solution of Dye-

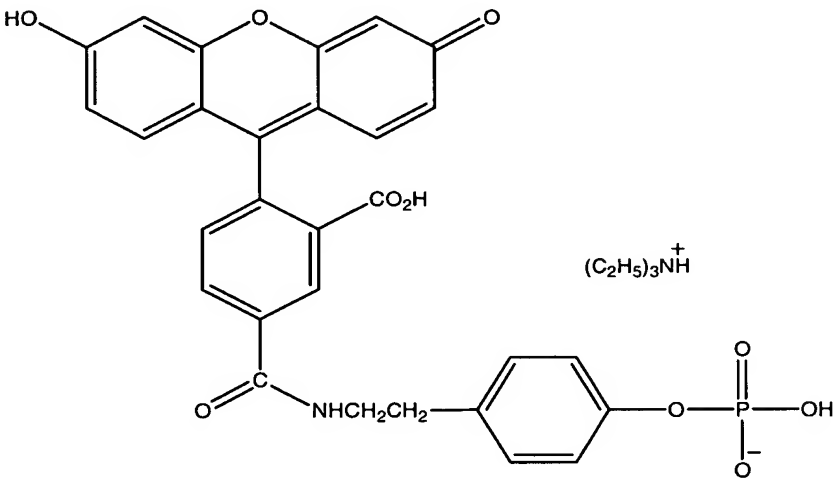
carboxylic acid, succinimidyl ester (6-isomer) (5mg, 0.01 mmol) in 500µl of DMF and the mixture is stirred at room temperature for 2 hours. To the reaction mixture is added ethyl acetate (10 ml) and stirred at room temperature for 5 minutes. The upper ethyl acetate layer is removed by decantation. The resulting aqueous layer is treated again with ethyl acetate (10 ml) and decanted. The remaining aqueous residue is purified by preparative TLC eluting with 20% H2O in acetonitrile. Obtained 4.0 mg of a pure desired product.



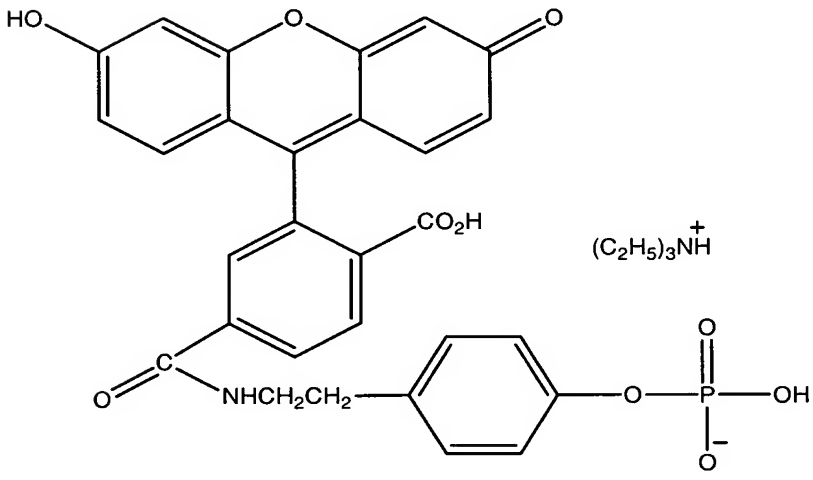
Compound **7**

This synthesis scheme was followed to produce Compounds **8-21**, wherein the same starting material was used but different reporter molecule compounds with a succinimidyl ester reactive group were conjugated to the O-Phosphotyramine intermediate. It is appreciated that numerous phosphotryamide ligand analogs can be made using this synthesis scheme wherein the desired reporter molecule with an appropriate reactive group

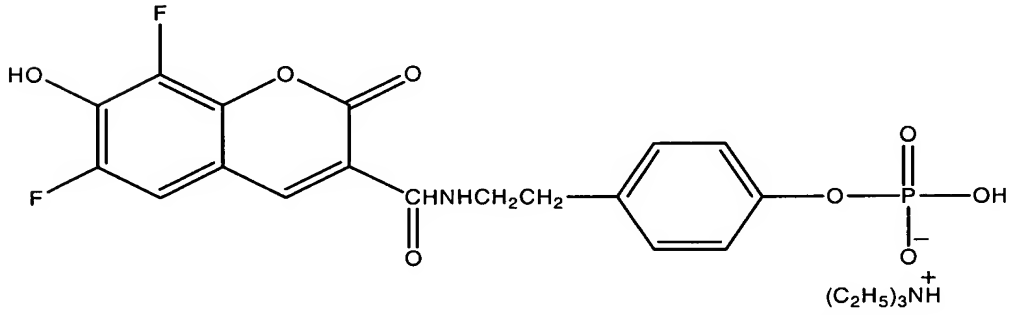
such as succinimidyl ester is conjugated to the phosphotyramide moiety. In this instance the following compounds are not intended to be limiting.



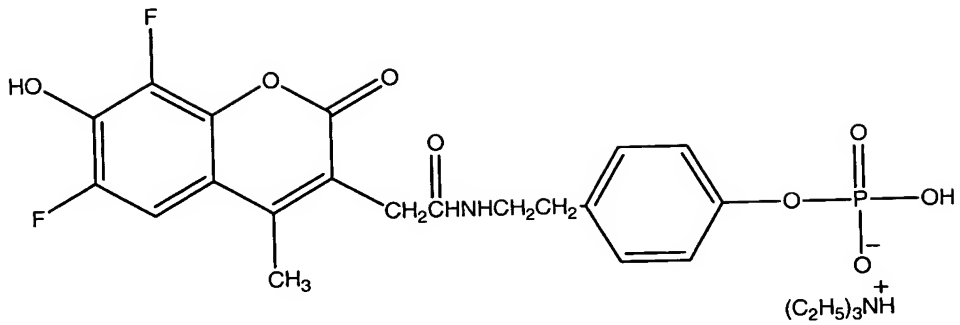
Compound **8**



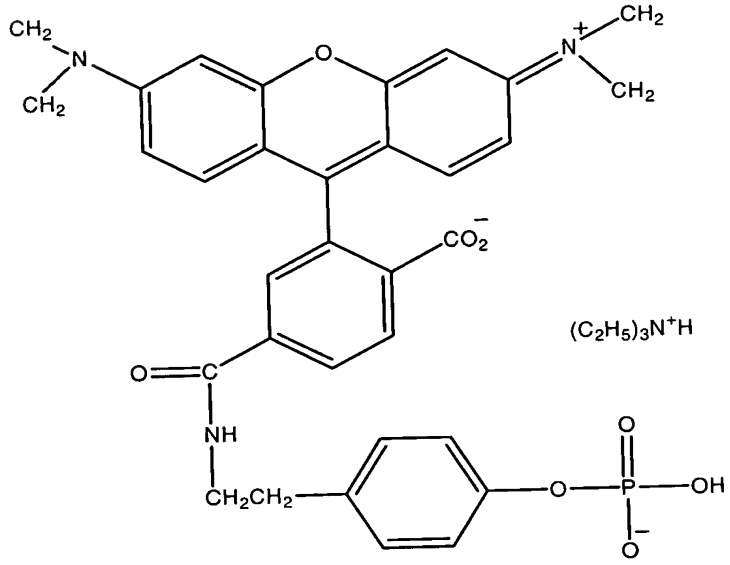
Compound **9**



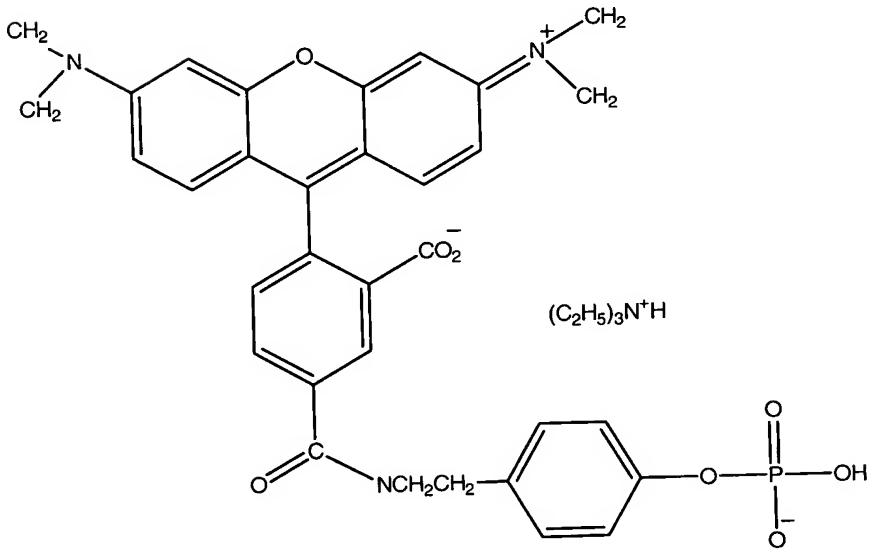
Compound **10**



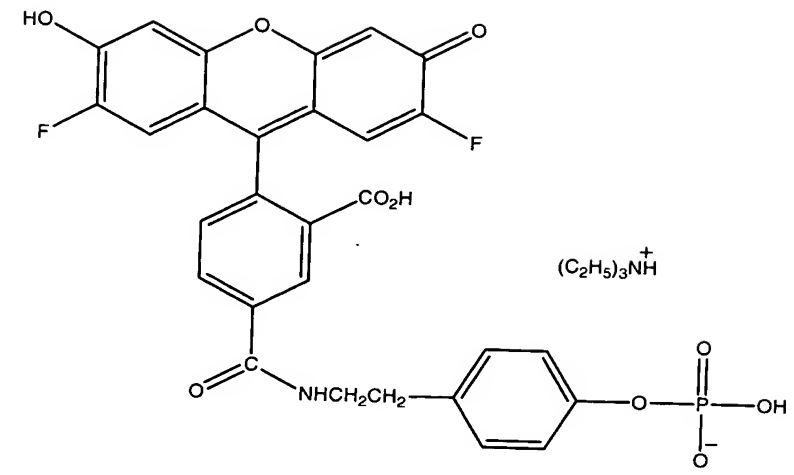
Compound **11**



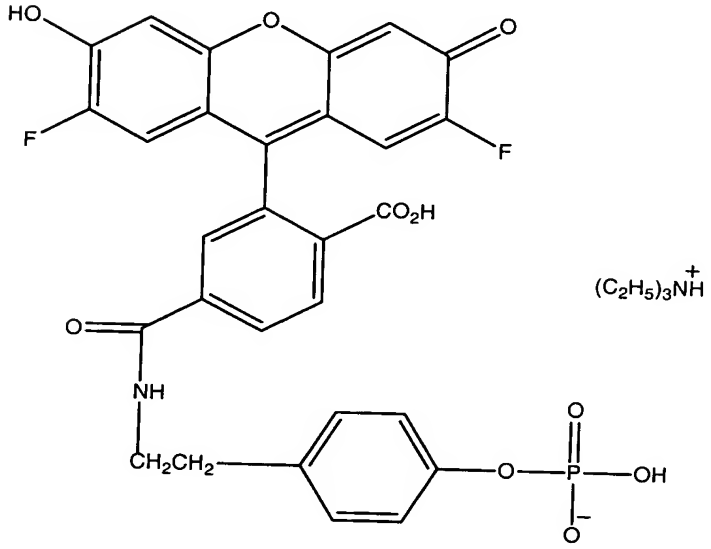
Compound **12**



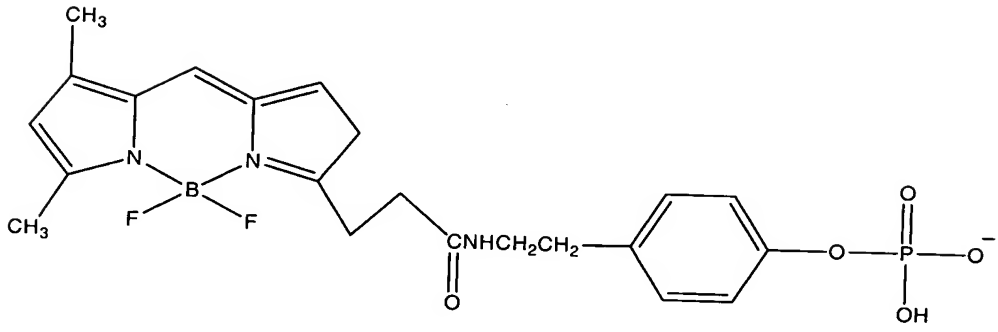
Compound **13**



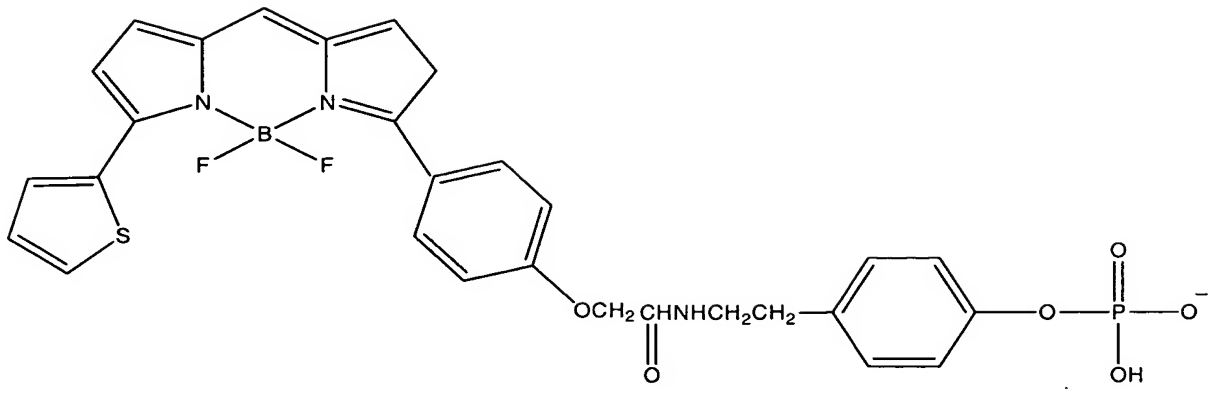
Compound **14**



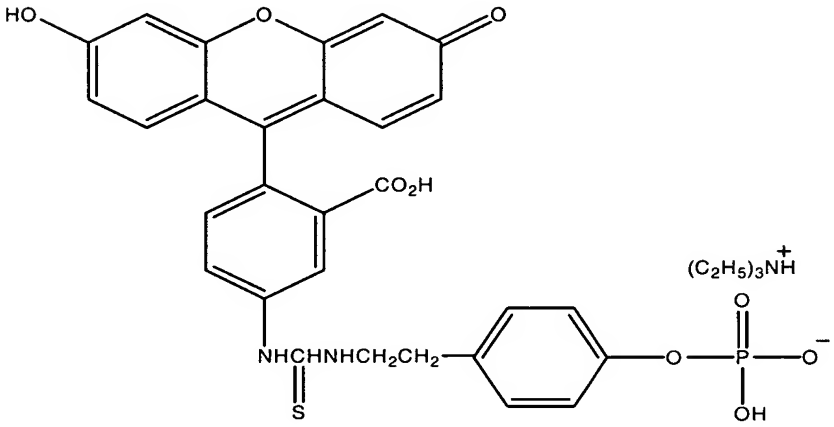
Compound **15**



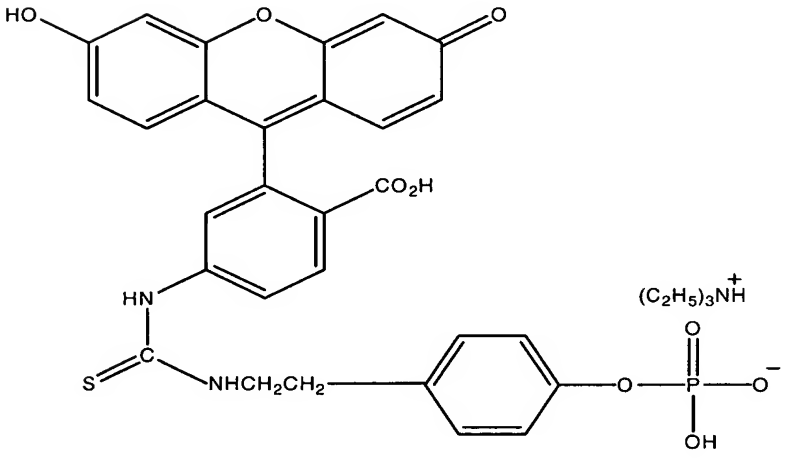
Compound **16**



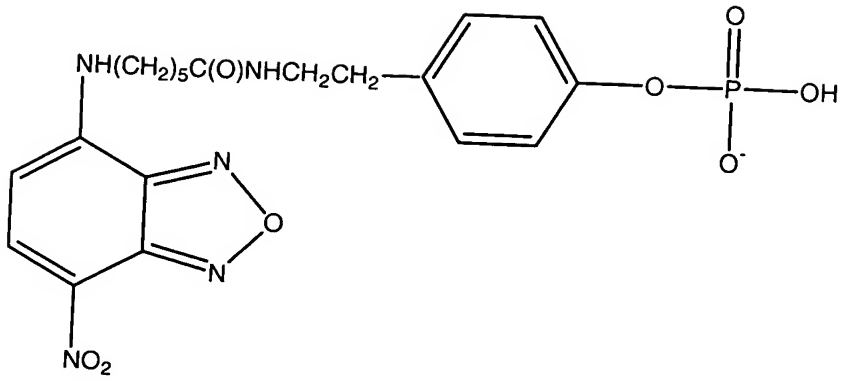
Compound **17**



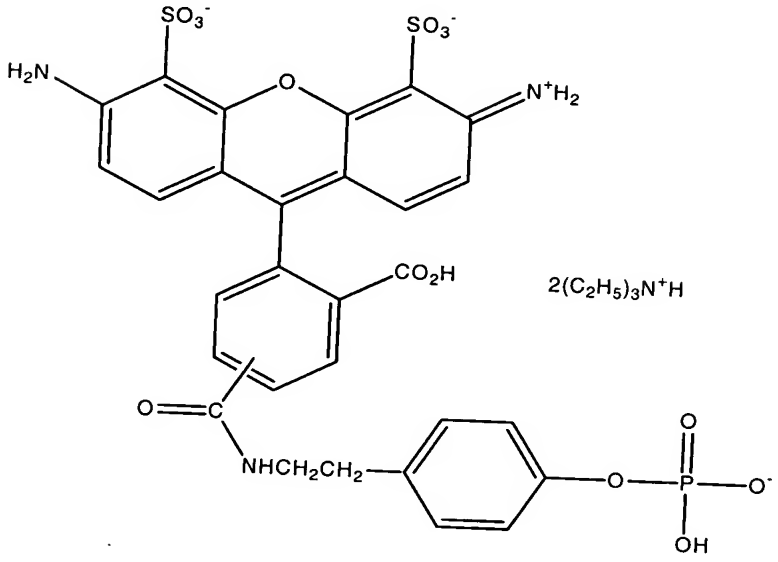
Compound **18**



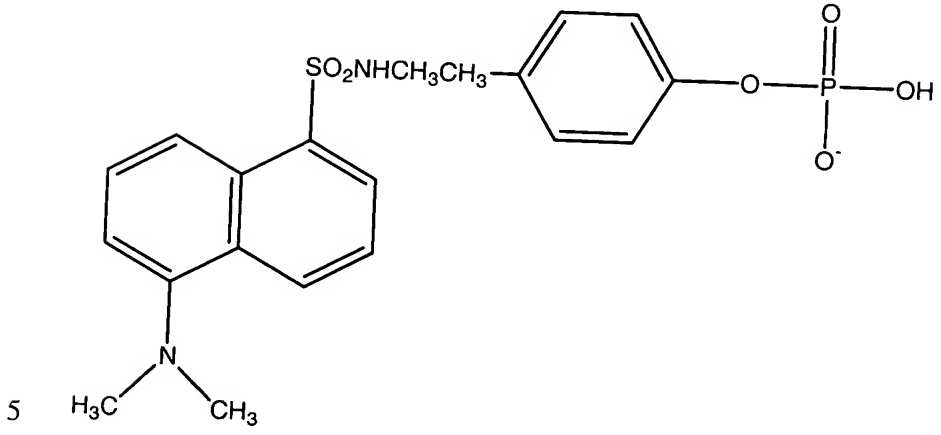
Compound **19**



Compound **20**



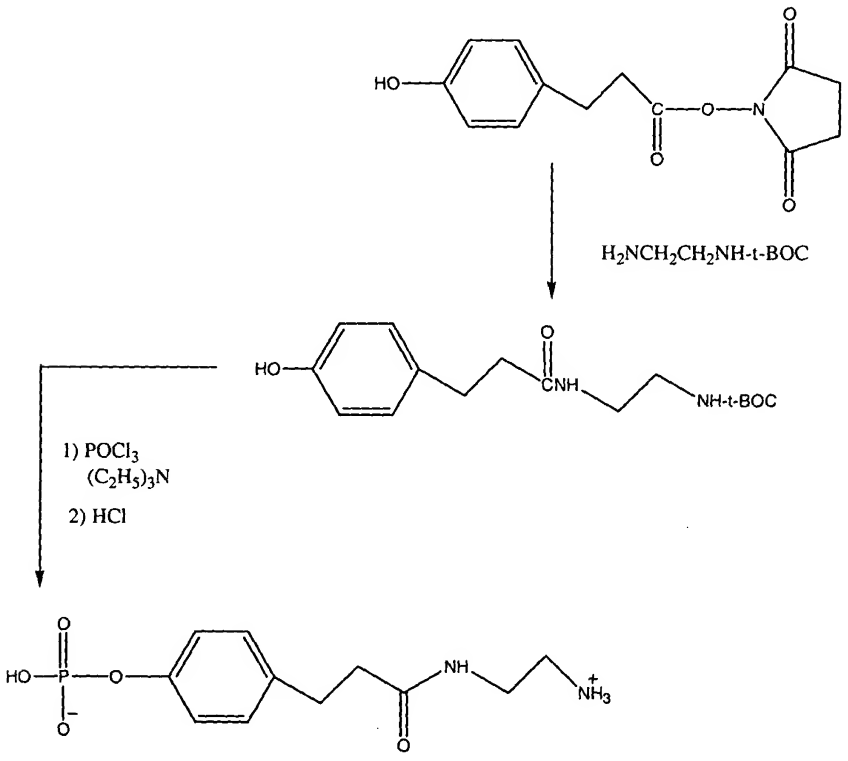
Compound **21**



Compound **39**

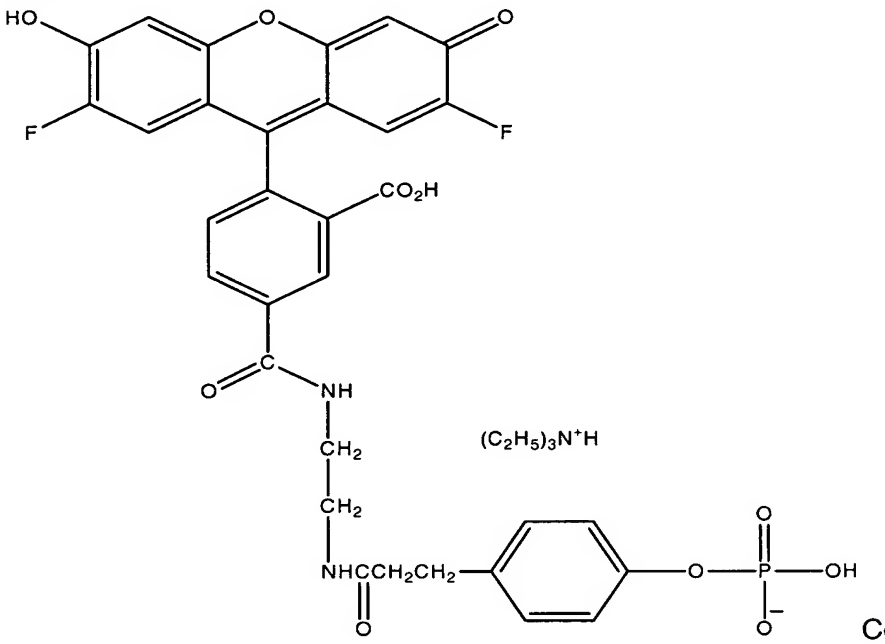
Exampl 27: Synthesis of homolog of phosphotyramide ligand analogs, Compounds **22-33** and **40**

The following compounds were made with the same starting material but different reporter molecule compounds with a succinimidyl ester reactive group. First, the phosphotyramide intermediate was synthesized wherein a solution of mono N-t_BOC ethylenediamine hydrochloride (1.0 g, 4.84 mmol) and triethylamine (1.35 ml, 9.66 mmol) in 100 ml of dichloromethane is added N-succinimidyl 3-(4-hydroxyphenyl)propionate (1.27 g, 4.82 mmol) and the mixture is stirred at room temperature for 6 hours. It is washed with 0.5% HCl (1x100 ml) and then with water (2x100 ml). The separed organic layer is dried over Na_2SO_4, filtered and concentrated under vacuum to give 1.0 g of an off-white solid. The resulting solid is dissolved in 30 ml of dichloromethane and cooled with and ice-water bath. To this ice-water cooled solution is added triethylamine (540 µl, 3.82 mmol), followed by addition of phosphorus oxychloride (340 µl, 3.71 mmol). After stirring at room temperature for 1 hour, all the solvent is removed under vacuum. To the resulting residue is added a solution of sodium bicarbonate (84 mg, 1 mmol) in 10 ml of water and stirred at room temperature overnight. From the combined desired fractions, homolog of O-Phosphotyramine is obtained.



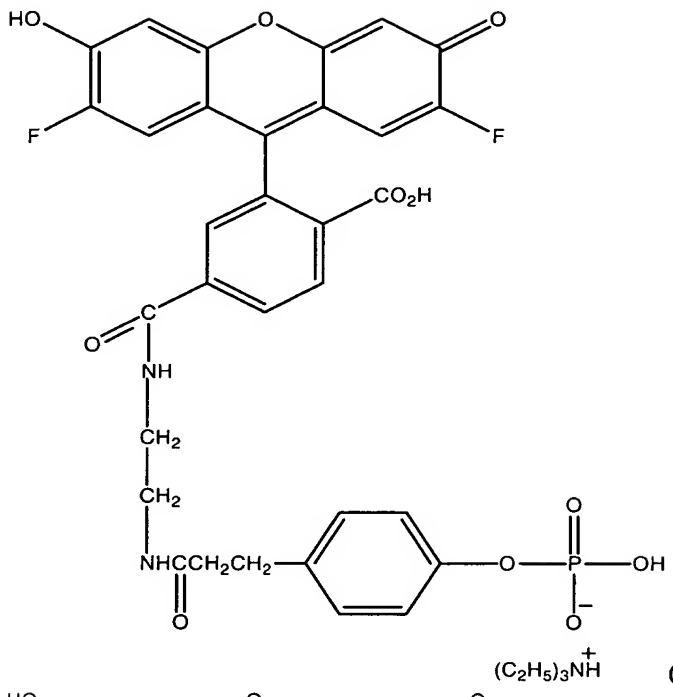
Next the succinimidyl ester version of the dye to homolog of O-Phosphotyramine was added to give Compound **22**. This is accomplished wherein a solution of the homolog of O-Phosphotyramine (3 mg, 0.01 mmol) and triethylamine (5µl, 0.04 mmol) in 500 µl of water is

added a solution of Dye-carboxylic acid, succinimidyl ester (6-isomer) (5mg, 0.01 mmol) in 500µl of DMF and the mixture is stirred at room temperature for 2 hours. To the reaction mixture is added ethyl acetate (10 ml) and stirred at room temperature for 5 minutes. The upper ethyl acetate layer is removed by decantation. The resulting aqueous layer is treated again with ethyl acetate (10 ml) and decanted. The remaining aqueous residue is purified by preparative TLC eluting with 20% H2O in acetonitrile. Obtained 4.0 mg of a pure desired product.

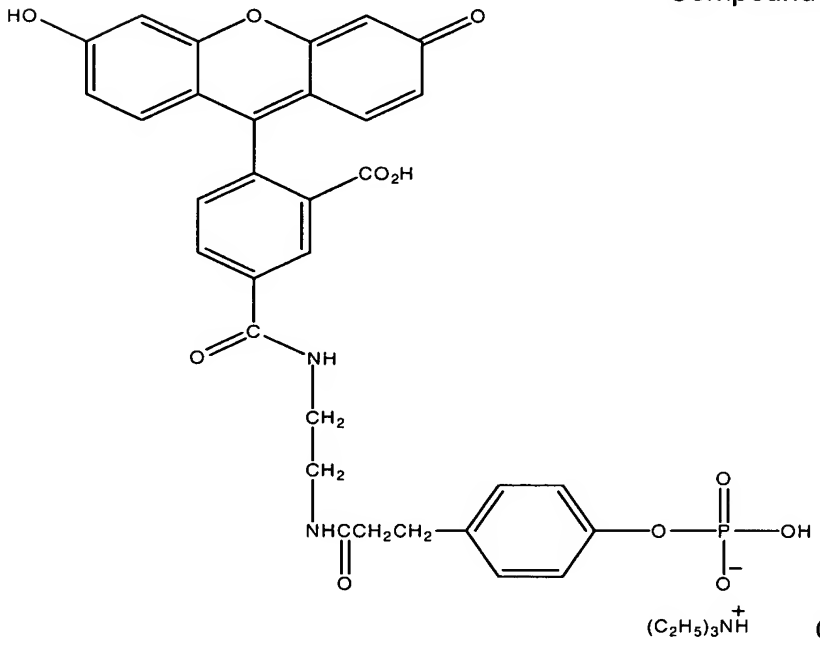


Compound **22**

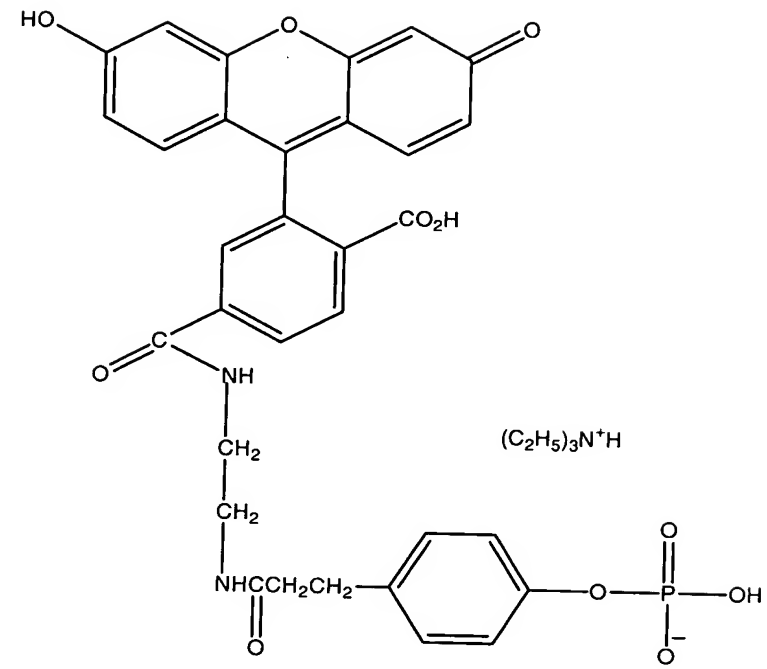
It is appreciated that numerous homologs of phosphotryamide ligand analogs can be made using this synthesis scheme wherein the desired reporter molecule with an appropriate reactive group such as succinimidyl ester is conjugated to the phosphotyramide moiety. In this instance the following compounds are not intended to be limiting. Compounds **23-33** and **40** were made using this synthesis scheme.



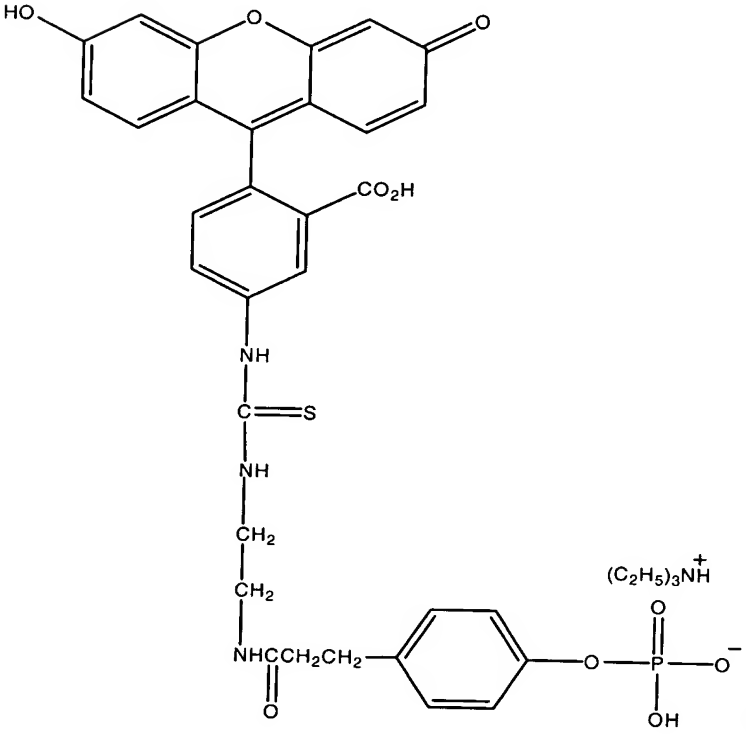
Compound **23**



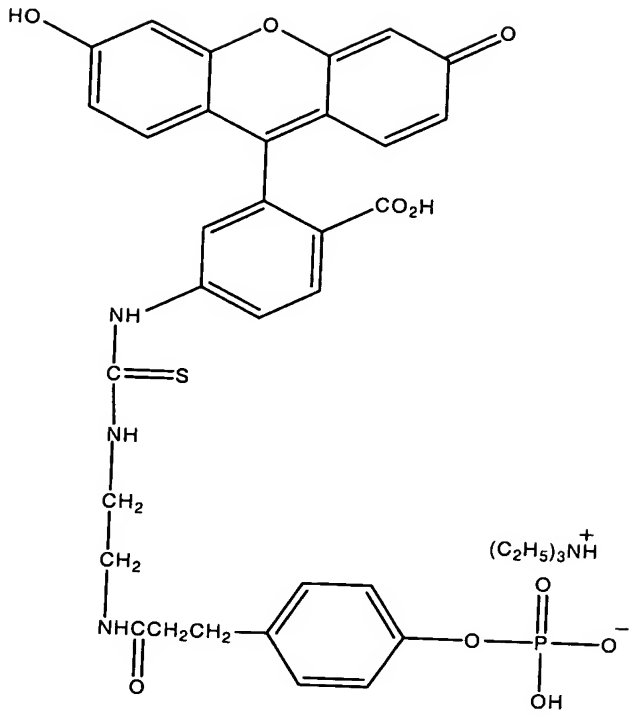
Compound **24**



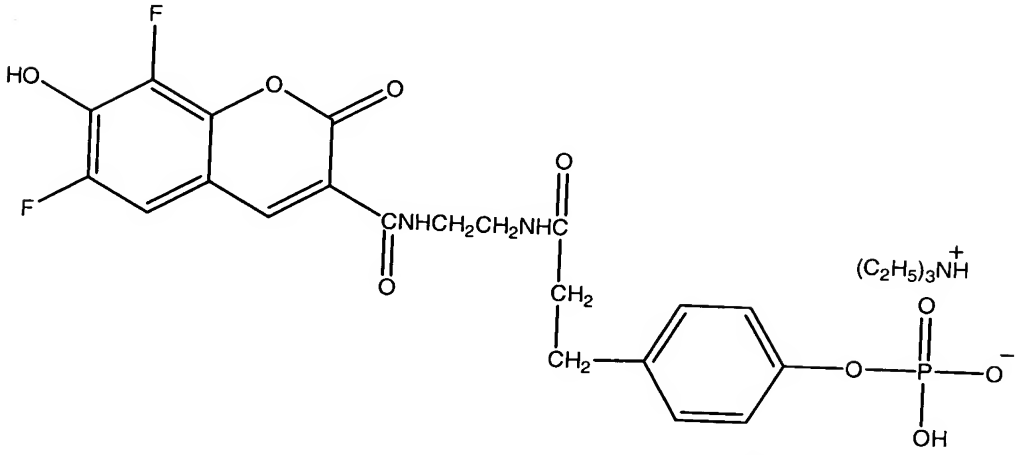
Compound **25**



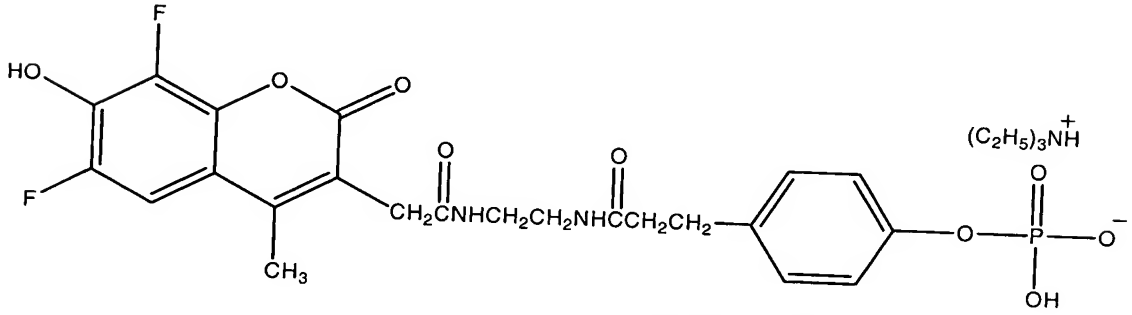
Compound **26**



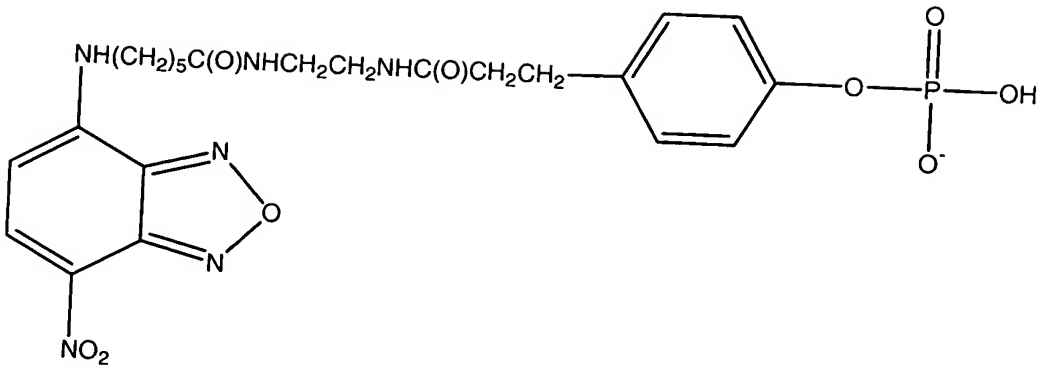
Compound **27**



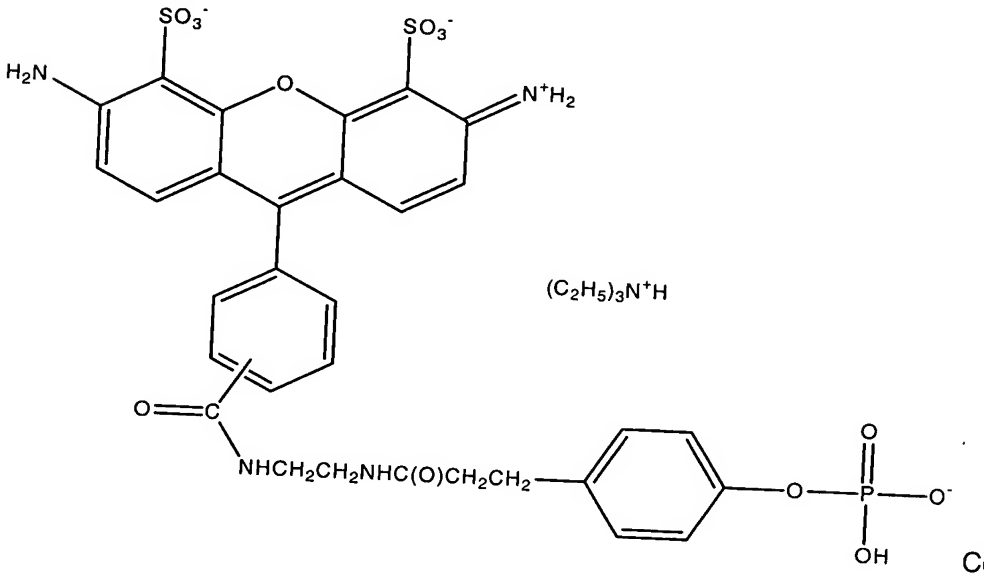
Compound **28**



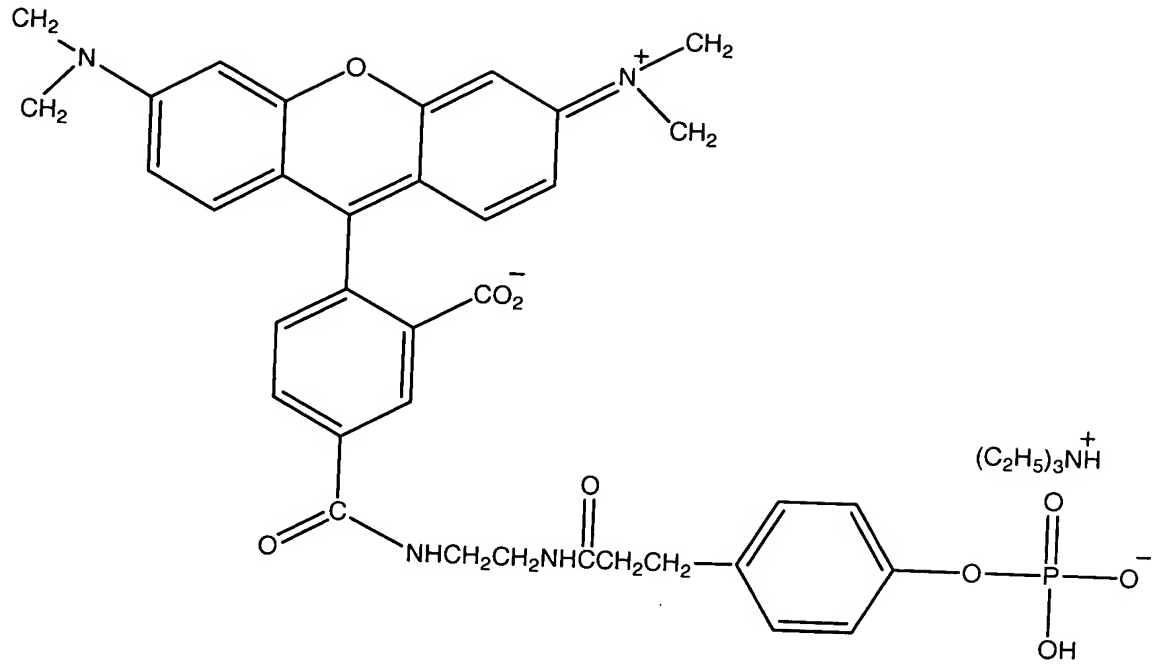
Compound **29**



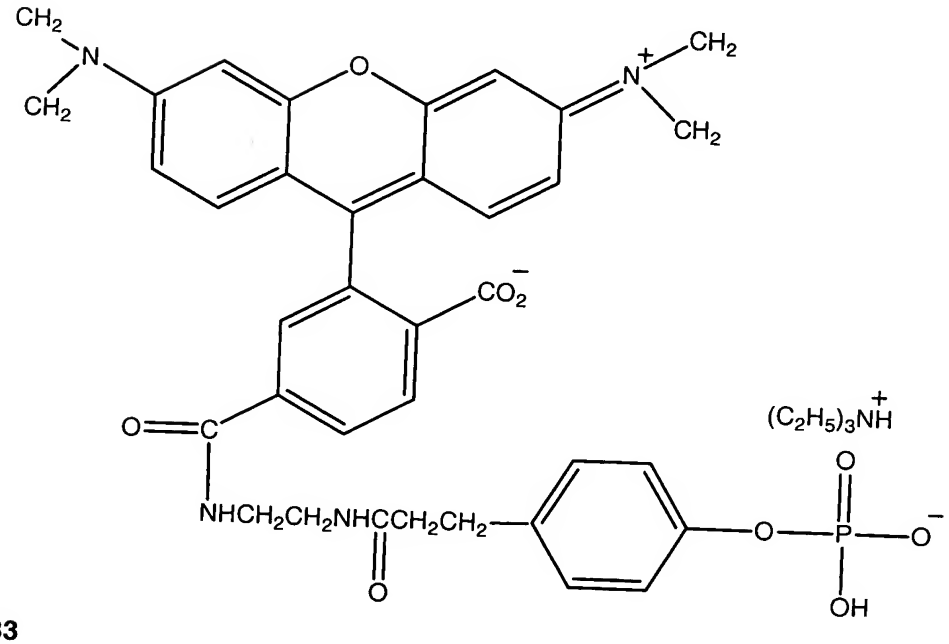
Compound **30**



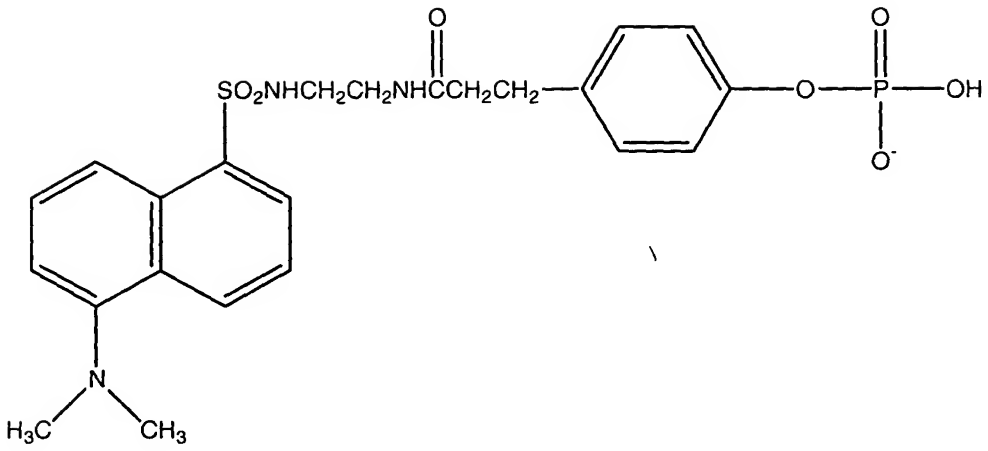
Compound **31**



Compound **32**



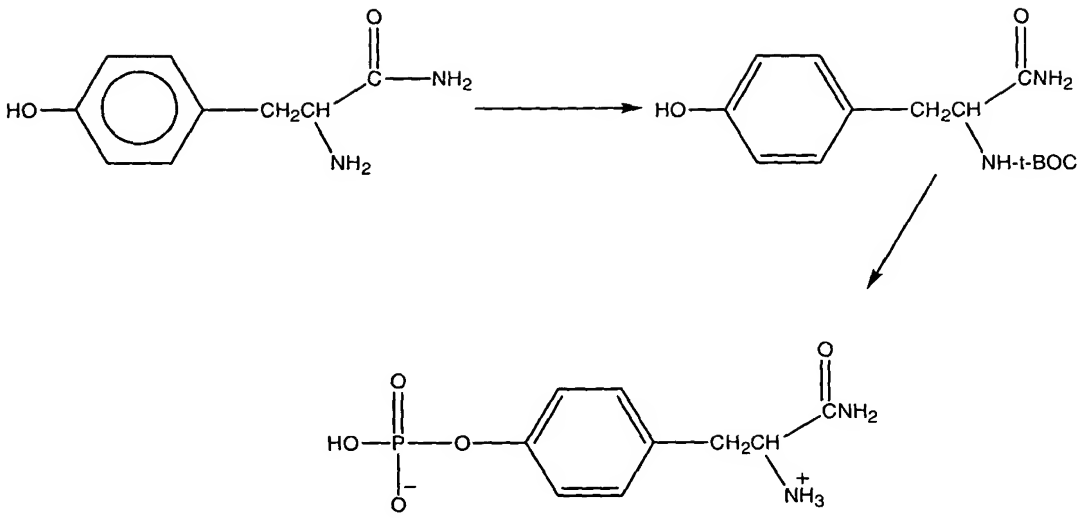
Compound **33**



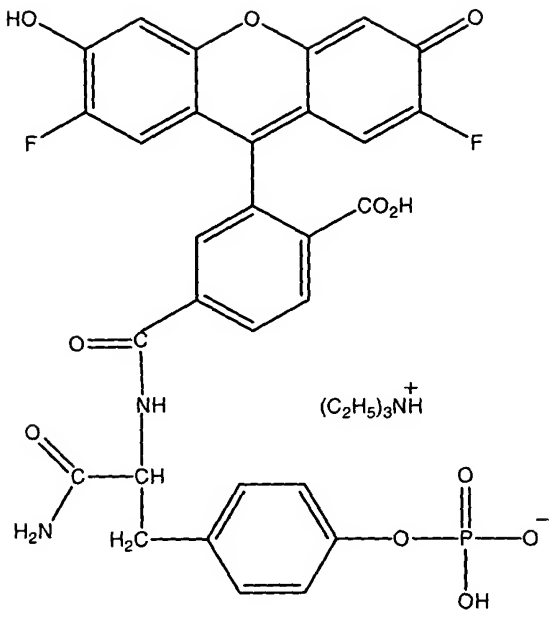
Compound **40**

Example 28: Synthesis of phosphotryosinamide ligand analogs, Compounds **34-38** and **41-42**

The following compounds were made with the same starting material but different reporter molecule compounds with a succinimidyl ester reactive group. First, the phosphotryosinamide intermediate was synthesized wherein a solution of L-tyrosinamide (0.75 g, 4.17 mmol) and triethylamine (640µl, 4.59 mmol) in 20 ml of THF was added di-tert-butyl dicarbonate (1.0 g, 4.59 mmol). After stirring at room temperature for 4 hours, 100 ml of chloroform was added and the mixture was washed with water (2x100 ml). The separated organic layer is dried over Na_2SO_4 and concentrated under vacuum to give 1 g of a white solid. This solid is dissolved in 30 ml of THF. To this solution is added di-tert-butyl diethylphosphoramidite (1.0 g, 3.93 mmol), followed by addition of tetrazole (750 mg, 10.70 mmol). After stirring at room temperature for 2 hours, a solution of 3-chloroperbenzoic acid (930 mg, 5.39 mmol) in 10 ml of dichloromethane was added while the reaction mixture was stirred under ice water bath. After stirring at room temperature for 1 hour, a 10% solution of sodium bisulfite in water (50 ml) was added and stirred at room temperature for 20 minutes. It was then extracted with chloroform (2x100 ml) and washed with 10% sodium bisulfite (2X100 ml) followed by washing with 10% sodium bicarbonate (1x100 ml). The separated organic layer was dried over sodium sulfate and concentrated under vacuum to give a crude fully protected intermediate. This crude intermediate was purified by column chromatography (silica gel) eluting with 5% methanol in chloroform to give 1.8 g of a protected intermediate. This is dissolved in 10 ml of TFA and stirred at room temperature overnight. All the TFA is removed under vacuum and the resulting residue is dissolved in about 2 ml of water and subjected to LH-20 column eluting with water. From the combined desired fractions 400 mg of a product is obtained as a white powder.

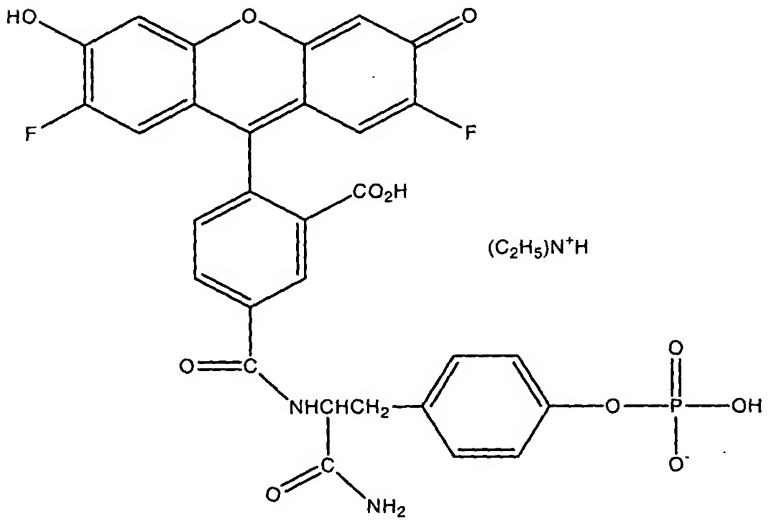


Next the succinimidyl ester version of a dye was added to phosphotryosinamide to give Compound **34**. This is accomplished wherein a solution of the phosphotryosinamide compound (3 mg, 0.01 mmol) and triethylamine (5µl, 0.04 mmol) in 500 µl of water is added to a solution of Dye-carboxylic acid, succinimidyl ester (6-isomer) (5mg, 0.01 mmol) in 500µl of DMF and the mixture is stirred at room temperature for 2 hours. To the reaction mixture is added ethyl acetate (10 ml) and stirred at room temperature for 5 minutes. The upper ethyl acetate layer is removed by decantation. The resulting aqueous layer is treated again with ethyl acetate (10 ml) and decanted. The remaining aqueous residue is purified by preparative TLC eluting with 20% H2O in acetonitrile. Obtained 4.0 mg of a pure desired product.

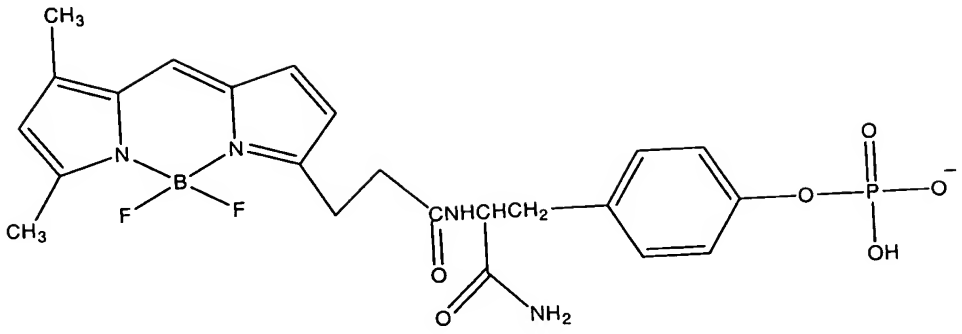


Compound **34**

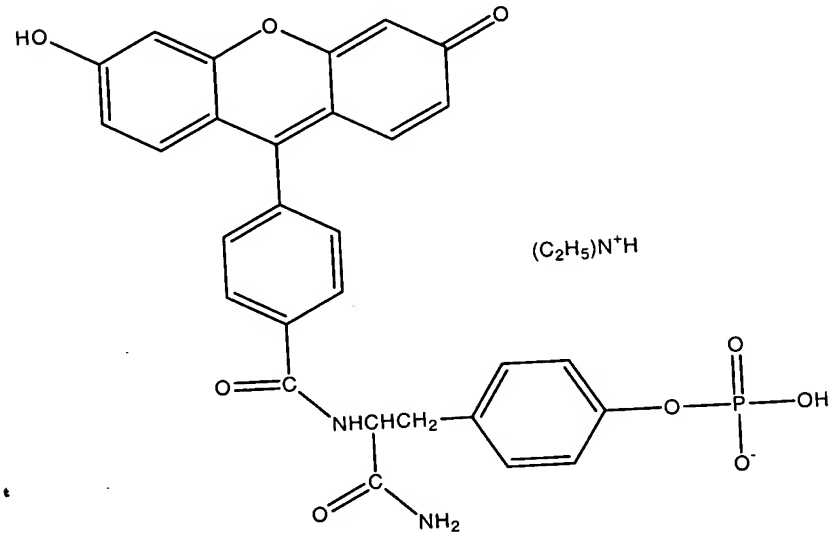
The following compounds were made with the same starting material but different reporter molecule compounds with a succinimidyl ester reactive group. It is appreciated that numerous phosphotryosinamide ligand analogs can be made using this synthesis scheme wherein the desired reporter molecule with an appropriate reactive group, such as succinimidyl ester, is conjugated to the phosphotryosineamide moiety. In this instance the following compounds are not intended to be limiting.



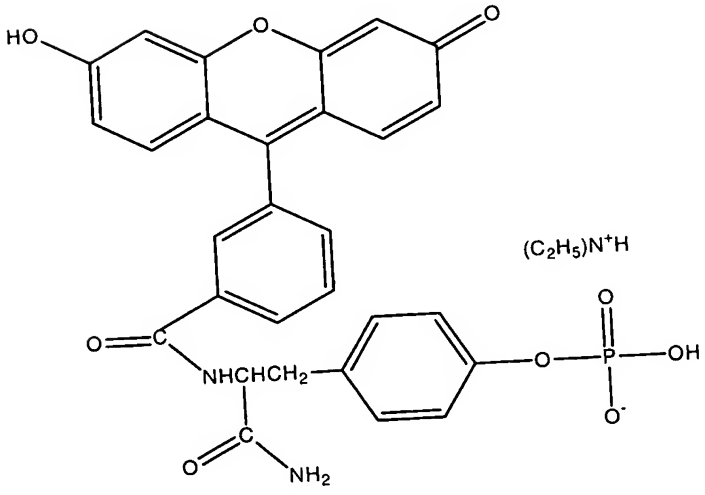
Compound **35**



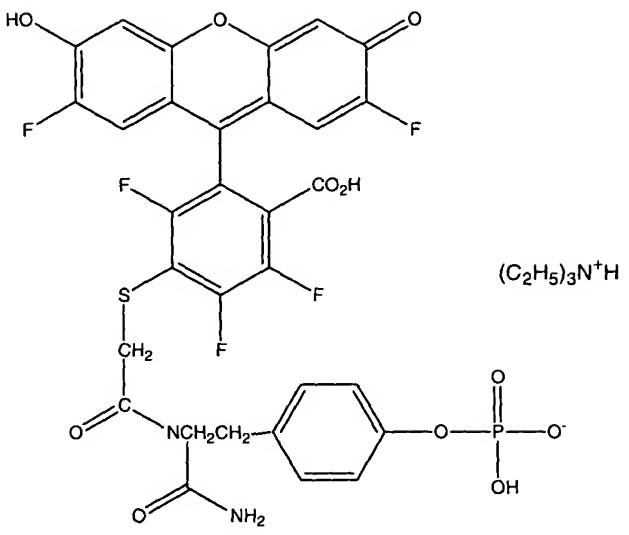
Compound **36**



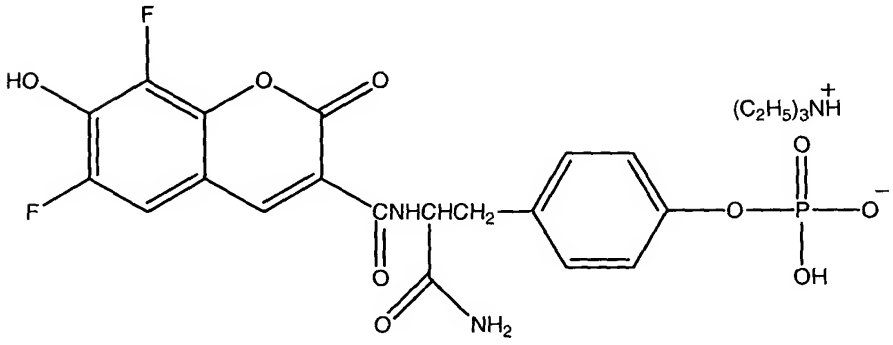
Compound **37**



Compound **38**



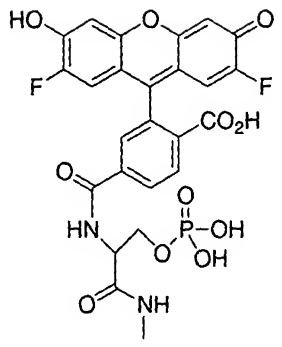
Compound **41**



Compound **42**

Example 29: Synthesis of phosphoserine ligand analog, Compound **43**

O-phosphorylserine, N-methylamide, N'-acylated with the 6-isomer of carboxy-2',7'-difluorofluorescein (B750-82-GEE45). The pH of a 0.10 M solution of O-phosphorylserine-N-methylamide was raised to 8.0 with aqueous sodium carbonate. A 100 µL aliquot (0.01 mmol) of this solution was added to a solution of Oregon Green 488 succinimidyl ester, 6-isomer (Molecular Probes 6149, 5.0 mg, 0.01 mmol) in 1.0 mL dioxane. The resulting mixture was stirred at room termpaerature for 4 hours, then filtered and lyophilized to afford Compound **43** as an orange powder.



Compound **43**

It is appreciated that other phosphoserine ligand analogs can be made using a similar synthetic scheme wherein a different reporter group with an appropriate reactive group such as succinimidyl ester is used to make numerous phosphoserine ligand analogs with various reporter groups. In this instance, Compound **43** is not intended to be limiting.

Example 30: Detection of Digoxigenin employing a ligand-binding antibody – labeling reagent – ligand analog ternary complex

To form a ligand-binding antibody labeling reagent complex, mouse monoclonal anti-Digoxigenin antibody (Roche product #1333062) in 50 mM MOPS buffer, pH 7.2, was mixed in a 1:5 ratio with a Fab fragment of goat-anti-mouse kappa chain labeled with a quenching moiety (QSY 9) (Molecular Probes, Inc.). The degree of QSY 9 labeling on the Fab fragment was 1.7, determined by absorbance. The ligand-binding antibody + labeling reagent complex (zero to 100 nm final concentration/zero to 500 nM final concentration) was serially diluted two-fold in 90 µl buffer down a black, 96-well flat-bottom microplate precoated with 1% (w/v) bovine serum albumin. As a control, the ligand-binding antibody (anti-Digoxigenin antibody) was diluted in the plate in 90 µl buffer. After the serial dilution, 10 µl of the ligand analog (BODIPY FL Digoxigenin, Molecular Probes Inc., B-23460) (50 nM final) in 50 mM MOPS buffer, pH 7.2 was added to the same wells.

The resulting fluorescence intensity was measured on a Victor^2 microplate reader (Wallac), 1 read/well for 1 sec each at 50000 V gain, excitation 485 +/- 17.5 nm, emission 535 +/- 12.5 nm. This demonstrates the ability of the quenching moiety on the labeling reagent to diminish the fluorescent signal of the BODIPY dye on the ligand analog when a ternary complex is formed. As the amount anti-Digoxigenin/Fab fragment complex increases, the fluorescence of the BODIPY-FL Digoxigenin decreases. After the initial read, 1 µl of 100 □M ligand (unlabeled Digoxigenin) (Sigma, catalog # D-9026) in 50 mM MOPS buffer, pH 7.2,

was added to all wells, and the resulting fluorescence intensity was measured on the same instrument at the same settings.

These results demonstrate that the ligand (unlabeled Digoxigenin) is capable of displacing the ligand analog to restore the fluorescent signal generated by the BODIPY fluorophore. When excess (1 μM) unlabeled Digoxigenin is added, the fluorescence quenching is partially relieved. *See,* Figure 9.

Example 31: Binding of ethanolamine phosphate ligand analog (Compound **2**) by anti-Akt antibody.

A 5 mM solution of BODIPY FL ethanolamine phosphate Compound **2** was made in water. A 2.5 mM solution of BODIPY FL ethanolamine (Compound **1**) was made in 50% (v/v) DMSO, *See* Example 1. Rabbit anti-Akt polyclonal antibody (Cell Signaling Technology, catalog # 9611) was serially diluted in 5 μl 50 mM Tris buffer, pH 7.5 in a black, 384-well flat-bottom Packard ProxiPlate preblocked with 0.25% (v/v) Mowiol. After the antibody was serially diluted in the plate, 5 μl of either Compound **2** or Compound **1** was added to the same wells. The final concentration of both compounds in the wells was each 50 nM. The final antibody concentration in the wells was zero to 250 nM. The fluorescence intensity was measured on an EnVision microplate reader (Perkin Elmer), PMT 1 gain 155, PMT 2 gain 191, excitation light 76%, 100 flashes at 9 mM height. The ligand analog, Compound **2**, is slightly quenched by the Rabbit anti-Akt polyclonal antibody when the ligand is bound by the antibody.

Example 32: Fluorescence enhancement of bis(acetamidophenylphosphate)–derivatized dye upon binding to antiphosphotyrosine antibody.

Solutions of Compounds **3**, **4** and **6** (100 nM) were prepared in 50 mM Tris-HCl, pH 7.5. 25 □l aliquots of these solutions were pipetted into the wells of a 384-well microplate. 25 μl aliquots of a serially-diluted 1 μM stock solution of P-Tyr-100 (US Patent No. 6,441,140) antiphosphotyrosine monoclonal antibody (Cell Signaling Technology, Beverly, MA) were added to the wells. The resulting samples contained 50 nM test compound and antibody concentrations ranging from 0.5 to 500 nM. Fluorescence intensity of the samples was measured on an EnVision microplate reader (PerkinElmer Life Sciences) using excitation/emission filter settings of 480/535 nm. The bis(acetamidophenylphosphate) (Compound **4**) exhibits fluorescence enhancement upon interaction with the antibody,

whereas the corresponding mono-substituted compound **6** and the parent bis(acetamidophenol) (Compound **3**) do not. See, Figure 10.

Example 33: Competitive Immunoassay with Compound **15** as the ligand analog, anti-phosphotyrosine antibody as the ligand-binding antibody and a phosphotyrosine peptide as the target ligand.

A 5 mM solution of Compound **15** was prepared in water. In 150 □l kinase buffer (50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 0.01% Brij 35, 2 mM DTT, 100 μM ATP) several separate reactions were made containing: 1) 100 nM Compound **15**; 2) 100 nM Compound 14 + 100 nM P-Tyr-100 monoclonal anti-phosphotyrosine antibody (Cell Signaling Technology, catalog # 9411); 3) 100 nM Compound **15** + 100 nM P-Tyr-100 + 10 □M phospho-pp60 c-src peptide (521-533) (TSTEPQY*QPGENL) from Bachem, catalog # H-3258; 4) 100 nM Compound **15** + 100 nM P-Tyr-100 + 10 □M phospho-abl peptide (EAIY*AAPFAKKK), custom peptide MPIJ6 from Anaspec. The resulting fluorescence was measured using a Hitachi F-4500 cuvette fluorimeter using 100 μl cuvettes. The F-4500 was set on 'emission scan', with an excitation at 470 nm, slit width of 5 nm, emission scan from 485-650 nm, slit width 5 nm, and the PMT gain was at 700 V.

Addition of P-Tyr-100 antibody to the ligand analog (Compound **15**) significantly quenched the reporter molecule of the ligand analog. Addition of either of two phosphotyrosine peptides relieved almost all of the quenching, demonstrating both the ability of the ligand-binding antibody to quench the ligand analog when bound to the antibody and the ability of the target ligand (phosphotyrosine peptide) to displace the ligand analog (Compound **15**). *See*, Figure 11

Example 34: Rapid displacement of the ligand analog by the target ligand

A 5 mM solution of Compound **15** was prepared in water. In 150 μl kinase buffer (50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 0.01% Brij 35, 2 mM DTT, 100 μM ATP) a reaction was made containing 100 nM Compound **15** + 100 nM P-Tyr-100 monoclonal anti-phosphotyrosine antibody (Cell Signaling Technology, catalog # 9411). The fluorescence was measured over time using a Hitachi F-4500 fluorimeter (Ex 470, Em 510, slit width 5nm for both wavelengths, PMT gain 700 V). After 15 seconds, 10 μM phospho-pp60 c-src peptide (521-533) (TSTEPQY*QPGENL) from Bachem, catalog # H-3258 was added.

The off rate of Compound **15** (ligand analog) was calculated as 0.14 sec^{-1}, demonstrating the ability of the target ligand to displace the ligand analog very rapidly. *See*, Figure 12.

Example 35:

A 2 mM solution of Compound **34** was prepared in water. Two working stocks were prepared in kinase buffer (50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 0.01% Brij 35, 2 mM DTT, 100 μM ATP): 1) 50 nM of Compound **34** + 100 nM P-Tyr-100 monoclonal anti-phosphotyrosine antibody (Cell Signaling Technology, catalog # 9411); 2) 100 □M phospho-pp60 c-src peptide (521-533) (TSTEPQY*QPGENL) from Bachem, catalog # H-3258. In two 384-well microplates, 25 μl of the 50 nM Compound **34** + 100 nM P-Tyr-100 complex was added to 96 wells in each plate. To the same wells, 25 □l of either kinase buffer alone (48 wells each plate) or the 100 μM phospho-pp60 c-src peptide in kinase buffer (48 wells each plate) was added. The fluorescence was measured in a Victor^2 microplate reader (Wallac), 1 read/well for 0.2 sec each at 30000V gain, excitation 485 +/- 17.5 nm, emission 535 +/- 12.5 nm.

The Z' statistic was calculated using equation 5 from Zhang, J.-H., Chung, T., D., Y., and Oldenburg, K. R. (1999) A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J. Biomol. Screen.* **4,** 67-73. The Z' factor using data from both plates combined was calculated as 0.919,demonstrating the ability to differentiate between background signal and signal generate when the target ligand is bound by the antibody. In other words, there is a 4-fold increase in fluorescent signal after the ligand analog is displaced by the phosphorylated peptide.

Example 36:

A 5 mM solution of Compound **15** was prepared in water. In kinase buffer (50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 0.01% Brij 35, 2 mM DTT, 100 □M ATP) 120 μM phospho-pp60 c-src peptide (521-533) (TSTEPQY*QPGENL) (Bachem, catalog # H-3258) was serially diluted three-fold across a microplate in 20 μl volume. 20 μl of 100 nM of Compound **15** + 200 nM P-Tyr-100 complex was added to all wells. The fluorescence intensity was measured on an EnVision microplate reader (Perkin Elmer), PMT 1 gain 155, PMT 2 gain 183, excitation light 60%, 100 flashes at 9 mM height.

To generate the Z' scores in Figure 7, the Z' factor was calculated using equation 5 from Zhang, J.-H., Chung, T., D., Y., and Oldenburg, K. R. (1999) A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J. Biomol. Screen.*

4, 67-73. This demonstrates the large increase in fluorescent signal after the ligand analog is displaced by the target ligand.

Example 37:

A 5 mM solution of Compound **15** was prepared in water. In kinase buffer (50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 0.01% Brij 35, 2 mM DTT, 100 µM ATP) 80 □M solutions of six different peptides or proteins were made: 1) phospho-pp60 c-src peptide (521-533) (TSTEPQY*QPGENL) (Bachem, catalog # H-3258); 2) non- phospho-pp60 c-src peptide (521-533) (TSTEPQYQPGENL) (Bachem, catalog # H-3256); 3) phospho-DSIP peptide (WAGGDAS*GE) (SynPep, catalog # 3920); 4) phospho-RRA(pT)VA peptide (RRAT*VA) (Sigma, catalog V248A); 5) beta-casein (Sigma, catalog # C6905); 6) bovine serum albumin (Sigma, catalog # A7284). These peptides or proteins were serially diluted 2-fold in 20 µl in a 0.025% Mowiol-blocked 384-well black Packard OptiPlate. 20 □l of a 2X mix of 50 nM of Compound **15** + 100 nM P-Tyr-100 (Cell Signaling Technology, catalog # 9411) complex in kinase buffer was added to the wells. The fluorescence intensity was measured on an EnVision microplate reader (Perkin Elmer), PMT 1 gain 155, PMT 2 gain 183, excitation light 60%, 100 flashes at 9 mM height.

The phospho-pp60 c-src peptide is the only peptide or protein that significantly displaces the ligand analog (Compound **15**), indicating that the reaction is specific for phosphotyrosine residues. See, Figure 13

Example 38: Displacement of phosphotyramide ligand analog by phosphotyrosine containing peptide but not by ATP

A 5 mM solution of Compound **15** was prepared in water. Separate reactions of 100 nM Compound **15** +/- 100 nM P-Tyr-100 (Cell Signaling Technology, catalog # 9411) complexes were made in kinase buffer with various amounts of ATP (50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 0.01% Brij 35, 2 mM DTT, with zero, 0.1, 0.25, 0.5, or 1 mM ATP). The fluorescence emission of each of the ten solutions was measured using a Hitachi F-4500 spectrofluorometer (Ex 450, Em 510, slit width 5nm for both wavelengths, PMT gain 700 V). After the initial read, 10 µM phospho-pp60 c-src (10 µM final concentration) peptide (521-533) (TSTEPQY*QPGENL) (Bachem, catalog # H-3258) in kinase buffer was added to the antibody:ligand reactions, and the resulting fluorescence intensity was measured on the same instrument at the same settings.

These data indicate that the assay is relatively insensitive to ATP concentrations. *See*, Figure 14.

Example 39: Detection of kinase activity

A 5 mM solution of Compound **15** was prepared in water. Abl kinase (New England Biolabs, catalog # P6050S) was serially diluted 2-fold in 20 □l kinase buffer (50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 0.01% Brij 35, 2 mM DTT, 500 μM ATP) in a 0.025% Mowiol-blocked 384-well black Packard OptiPlate. To the wells containing the serial dilution, 20 □l of either 400 μg/ml poly (Glu:Ala:Tyr) 6:3:1 ratio (Sigma, catalog # P-3899) + 100 nM p-Tyr-100 antibody (Cell Signaling Technology, catalog # 9411) + 50 nM Compound **15** in kinase buffer or 100 μM abl substrate peptide (custom peptide from AnaSpec, MPIJ-5 (EAIYAAPFAKKKC)) + 100 nM p-Tyr-100 antibody + 50 nM Compound **15** in kinase buffer was added. After a one hour incubation the fluorescence intensity was measured on a Victor^2 microplate reader (Wallac), 1 read/well for 0.1 sec each at 20000 V gain, excitation 450 +/- 3 nm, emission 510 +/- 20 nm.

The assay is capable of detecting Abl kinase activity wherein the phosphorylated peptides displace the ligand analog (Compound **15**). The Abl kinase can phosphorylate both peptides, though it is more effective at phosphorylating the abl substrate peptide, MPIJ-5. See, Figure 15.

Example 40:

A 5 mM solution of Compound **15** was prepared in water. In kinase buffer (50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 0.01% Brij 35, 2 mM DTT, 100 μM ATP) prepared 150 μl 333.3 μM staurosporine (Sigma, catalog # S-4400). The 333.3 μM staurosporine stock was serially diluted three-fold in 15 μl kinase buffer in a 0.025% Mowiol-blocked 384-well black Packard OptiPlate. To the same wells 10 μl of either kinase buffer alone, 10 μl of 333 Units/ml abl kinase (New England Biolabs, catalog # P6050S) in kinase buffer, or 10 μl of 125 Units/ml src kinase (Upstate Biotechnology, catalog # 14-326) in kinase buffer was added. The plate was centrifuged to ensure mixing and then incubated for 20 minutes at 37°C. To this, 25 μl of kinase buffer containing 200 nM Compound **15** + 200 nM p-Tyr-100 antibody (Cell Signaling Technology, catalog # 9411) + 1.2 μM Fab fragment of goat-anti-mouse antibody labeled with Alexa Fluor® 555 as a quenching moiety + 250 μg/ml poly (Glu:Tyr) 4:1 ratio (Sigma, catalog # P-0275). The plate was centrifuged again then incubated at 37°C while monitoring the fluorescence intensity in a Victor^2 microplate reader

(Wallac), 1 read/well for 0.1 sec each at 20000 V gain, excitation 450 +/- 3 nm, emission 510 +/- 20 nm. After 45 minutes there was no increase in intensity in the abl-kinase-containing wells, so abl peptide substrate (custom peptide from AnaSpec, MPIJ-5 (EAIYAAPFAKKKC) was added to a final concentration of 100 μM, and the plate incubated for an additional 50 minutes at 37°C.

The assay can determine the IC_{50} of staurosporine, a model tyrosine kinase inhibitor. This indicates that the assay can be used to determine the IC_{50} of unknown/experimental tyrosine kinase inhibitors. *See*, Figure 16.

Example 41:

A 4 mM stock of Compound **16** was made in 16% (v/v) DMSO. Three different antibodies were diluted to 1 μM in 50 mM MOPS, pH 7.2: 1) p-Tyr-100 antibody (Cell Signaling Technology, catalog # 9411); 2) p-Tyr-69 antibody (BD Transduction Labs, catalog # 610430); 3) p-Tyr-20 antibody (BD Transduction Labs, catalog # 610000). A fourth antibody, the 4G10 antibody (Upstate Biotechnology, catalog # 05-321), was diluted to 400 nM in MOPS buffer. The antibodies were serially diluted in a microplate in 25 □l 50 mM MOPS, pH 7.2, buffer. 25 μl 100 nM Compound **16** in MOPS buffer was added to all wells. The fluorescence intensity was measured on an EnVision microplate reader (Perkin Elmer), PMT 1 gain 155, PMT 2 gain 187, excitation light 53%, 100 flashes at 9 mM height.

This experiment indicates that the p-Tyr-100 antibody from Cell Signaling Technology has the highest affinity for the ligand analog (Compound **16**). *See*, Figure 17.

Example 42: O-Phosphotyrosine causes minimal displacement of phosphotyramide–dye ligand analogs from antibody binding sites

Solutions containing 100 nM ligand analog Compound **15** or ligand-detection complex (100 nM (B573-85-HCK) + 100 nM P-Tyr-100 antiphosphotyrosine monoclonal antibody (Cell Signaling Technology, Beverly, MA)) were prepared in 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 0.01% Brij-35, 100 μM ATP, 2 mM DTT, 1 mM EGTA. Fluorescence emission spectra of these samples were recorded on a Hitach F-4500 spectrofluorometer using an excitation wavelength of 470 nm. *O*-phosho-L-tyrosine (Sigma Chemical Co., St. Louis, MO) or phosphotyrosine peptide (phospho-pp60 c-src (521–533); TSTEPQY*QPGENL, Bachem California, Inc. Torrance, CA) at a concentration of 10 μM were added to samples of the detection complex and the resulting change in fluorescence was measured.

Example 43: Method for selecting ligands for use in displacement assay

Table 4. Ligands screened for efficacy in Figures 14A–D

Compound Number	Ligand analog	F_{max}/F_{min}**
15	Oregon Green 488 6-phosphotyramide	5.4
7	Oregon Green 514 phosphotyramide	4.0
8	5-FAM phosphotyramide	1.3
9	6-FAM phosphotyramide	3.2
18	5-FITC phosphotyramide	1.7
19	6-FITC phosphotyramide	1.6
**obtained from data in Figure B as the ratio of the fluorescence intensity at the lowest antibody concentration (F_{max}) to that at the highest antibody concentration (F_{min}).		

Solutions of test compounds (100 nM) were prepared were prepared in 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 0.01% Brij-35, 500 µM ATP, 2 mM DTT, 1 mM EGTA. 20 µl aliquots of these solutions were pipetted into the wells of a 384-well microplate. 20 µl aliquots of a serially-diluted 0.8 µM stock solution of P-Tyr-100 antiphosphotyrosine monoclonal antibody (Cell Signaling Technology, Beverly, MA) were added to the wells. The resulting samples contained 50 nM of each ligand analog (Compounds **15, 7, 8, 9, 18** and **19**) and antibody concentrations ranging from 0.8 to 500 nM. Fluorescence polarization and intensity of the samples was measured on an EnVision microplate reader (PerkinElmer Life Sciences) using excitation/emission filter settings of 480/535 nm. Increasing fluorescence polarization as a function of antibody concentration (A) provides confirmation of ligand analog binding to the antibody. Ligand analogs exhibiting the largest possible signal changes (B) upon antibody binding (i.e. largest value of F_{max}/F_{min} see Table 4) are preferred for displacement assays using a fluorescence intensity readout. Phosphotyrosine peptide, target ligand, (phospho-pp60 c-src (521–533); TSTEPQY*QPGENL, Bachem California, Inc. Torrance, CA) was then added to all samples at a concentration of 10 µM and the fluorescence intensity and polarization measurements were repeated (C, D). Preferred ligand analogs for displacement assays using a fluorescence intensity readout exhibit large fluorescence intensity upon phosphopeptide addition (i.e. C compared to B). Displacement of the ligand analogs from antibody is confirmed by depolarization of fluorescence (D compared to A). Based on these considerations, xanthene dye-based ligand analogs with a phosphotyramide moiety attached at the 6-position of the carboxyphenyl ring (e.g Compound **8**) exhibit superior performance to the corresponding compounds derivatized at the 5–position (e.g. Compound **9**). See, Figure 18.

Example 44: Comparison of phosphotyramide ligand analog to phosphotyrosinamide ligand analog: Determination of dissociation constants for antibody–ligand complexes.

Solutions of Compound **15** and Compound **34** (2 nM) were prepared in 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 0.01% Brij-35, 500 µM ATP, 2 mM DTT, 1 mM EGTA, 0.5 mg/ml bovine serum albumin. 25 µl aliquots of these solutions were pipetted into the wells of a 384-well microplate. 25 µl aliquots of a serially-diluted 0.08 µM stock solution of P-Tyr-100 anti-phosphotyrosine monoclonal antibody (Cell Signaling Technology, Beverly, MA) were added to the wells. The resulting samples contained 1 nM test compound and antibody concentrations ranging from 0.02 to 40 nM. Fluorescence intensities of triplicate samples at each antibody concentration were measured on an EnVision microplate reader (PerkinElmer Life Sciences) using excitation/emission filter settings of 480/535 nm. The mean (n = 3) fluorescence intensities were plotted against the corresponding antibody concentrations. Dissociation constants were determined from hyperbolic single-site saturation binding functions fitted to the experimental data by nonlinear regression analysis (SigmaPlot, Jandel Scientific Inc). The dissociation constants obtained were 2.0 nM for Compound **15** + P-Tyr-100 and 1.7 nM for Compound **34** + P-Tyr-100.

Example 45: Detection of a target ligand

Compound **12** and Compound **13** were assessed for efficacy using the methods described in Example 43. The results of this assessment show large fluorescence polarization changes (Figure 20A), indicating binding of the ligands to the P-Tyr-100 antibody, but little or no analytically useful fluorescence intensity change (Figure 20B). The experiment shown in Figure 20C demonstrates that complexation of the mouse monoclonal anti-phosphotyrosine antibody (P-Tyr-100) with a secondary antibody labeled with a fluorescence resonance energy transfer acceptor dye (Alexa Fluor 647 dye–labeled $F(ab')_2$ fragment of goat anti-mouse IgG; "labeling reagent") results in quenching of the fluorescence of Compound **13** that is reversed up addition of a phosphotyrosine-containing peptide. Samples containing 100 nM of Compound **13** and additional components identified in the figure legend were prepared in 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 0.01% Brij-35, 100 µM ATP, 2 mM DTT, 1 mM EGTA. 100 µL volumes of these samples were transferred to microcuvettes. The fluorescence emission spectrum was recorded for each sample using a Hitachi F-4500 spectrofluorometer (excitation wavelength = 520 nm). *See*, Figure 19.

Example 46: Flow Cytometric Assays

Coupling of antibodies to microspheres is a common methodology utilized to develop assays for analytes detected using flow cytometry instrumentation. Antibody-microsphere coupling procedures are typically performed so as to preserve antigen-binding activity. Hence, antibodies on microspheres will be capable of binding the ligand analog. Upon exposure of antibody-coupled microspheres to antigen, the ligand analog will be displaced from the microsphere, and a signal decrease will be observed in the flow cytometer. To generate flow cytometry assays with signals that increase upon analyte detection, a bound labeling reagent (anti-Fc antibody fragment) can be complexed with the microsphere-coupled ligand-analog bound antibody. The observed signal from the labeling reagent will be quenched, through FRET to the ligand-analog. Upon exposure to the antibody-coupled microsphere to antigen, the ligand analog will be displaced, and the donor labeling reagent signal will increase.

Example 47: The use enzyme amplified detectable signal with an enzyme cofactor conjugated ligand analog for a one step immunoassay.

The fluorophore moiety of the ligand-detection-reagent is replaced with an "enzyme-activating-factor" and then the signal associated with a single antigen-binding event is enzymatically amplified. For instance, the cofactor-ligand-analog is pre-complexed with the antibody wherein the ligand analog is displaced upon antigen binding. The types of cofactors utilized are: NAD(P)H, ATP, GTP, cAMP, coenzyme-A, FADH, hematin, etc. Any small molecule that can be coupled to an antigen and still retain the ability to activate an enzyme-reaction are candidates for this approach. Antigen target detection is performed in the presence of the inactive (cofactor requiring) enzyme. The concentrations of the reagents are established, such that while the cofactor-ligand-analog is bound by antibody, the cofactor-requiring enzyme is not able to bind cofactor (not effective at competing with the antibody). Upon displacement of the cofactor-ligand-analog from the antibody, the cofactor-requiring enzyme binds the cofactor-ligand-analog and is activated. Any fluorogenic or chromogenic substrate is utilized that can be coupled to the activated enzyme and thereby significantly amplifying the single antigen-binding event. In this manner, the requirement for a second separate detection/amplification antibody to detect the antigen is obviated,

Example 48: Efficacy of secondary antibody labels as fluorescence resonance energy transfer acceptors.

Table 5.

Dy lab l on Fab	Sample A.	Sample B.	Sampl C.

(DOS)**	100 nM Compound 16	100 nM Compound 16 + 100 nM P-Tyr-100	100 nM Compound 16+ 100 nM P-Tyr-100 + 600 nM Fab
Unlabeled	100	51	51
Alexa Fluor 555 (3.8)	100	53	36
Alexa Fluor 594 (2.5)	100	49	48
Alexa Fluor 647 (2.8)	100	49	47
Tabulated values are fluorescence intensities measured at 510 nm (excitation at 450 nm) expressed as percentages of the fluorescence intensity of 100 nM phosphotyramide ligand analog (Sample A) under the same conditions. **DOS = degree of substitution i.e. the average number of dye labels per antibody.			

Solutions containing (A) 100 nM phosphotyramide ligand analog, (B) 100 nM phosphotyramide ligand analog complexed with 100 nM P-Tyr-100 mouse monoclonal anti-phosphotyrosine antibody and (C) 100 nM phosphotyramide ligand analog complexed with 100 nM P-Tyr-100 anti-phosphotyrosine antibody and 600 nM Fab fragments of goat anti-mouse IgG were prepared in 50 mM Tris-HCl, pH 7.5. Corresponding sets for solutions were prepared for Fab fragments labeled with three different dyes and an unlabeled control. Fluorescence intensities at 510 nm (excitation at 450 nm) were measured on a Hitachi F-4500 spectrofluorometer and were expressed as percentages of the intensity of the free phosphotyramide ligand sample (Table 5). In all cases, binding of the ligand to the P-Tyr-100 antibody resulted in an approximately 50% decrease of fluorescence intensity. Addition of a labeled secondary antibody produced a further decrease in intensity only in the case of the Alexa Fluor 555 labeling dye, which has spectral characteristics that are consistent with efficient fluorescence resonance energy transfer from the BODIPY FL phosphotyramide ligand (Compound **16**).

Example 49: The use of ligand-detection reagent with protein microarray assays.

Antibodies are immobilized on a solid surface to form a microarray. Antibody arrays of this type are often utilized for analyte detection in a multi-step process. The microarrayed antibody captures the antigen, and then a second detection antibody is utilized to record the presence/absence of the captured antigen. Utilizing ligand-detection-reagents, this multi-step process can be eliminated, and antigen detection performed in the following manner. Microarraying antibodies either pre-bound with ligand-analog or subsequently exposed to ligand-ligand, will generate a signal associated with the bound ligand. Exposure of that antibody to a detectably distinct labeling reagent, generates two independent signals associated with any given microarrayed antibody spot. Exposure of the protein microarray to antigen (target ligand) will cause displacement of the ligand-analog signal, but will not alter the labeling reagent signal. For instance, for phosphotyrosine detection, a high affinity

binding (yet not quenching) dye-antigens are utilized such as compound **12** or **13**. Microarraying the antiphosphotyrosine-Compound **12** complexes will yield "bright-spots" on the protein microarray when detected with the appropriate excitation/emission filters. For graphical representation purposes, these data can be psuedo-colored "green." Pre-complexing the same antibodies with, for instance, a detectably distinct labeling reagent will yield bright-spots when detected with the appropriate excitation/emission filters. For graphical representation purposes, these data can be psuedo-colored "red." In the absence of antigen, the images psuedo-colored "red" and "green" can be superimposed (overlay image) to generate a "yellow" spot, indicative of the absence of antigen. Exposure of the antibody microarray to target ligand will cause a displacement of the "green" signal and not effect the "red" signal. Hence, a single-step determination of the exposure of the antibody microarray to target ligand can be quantitated by observing to what degree the original "yellow-spots" change to "red-spots." For 100% displacement, pure red-spots would be observed, and for 0% analyte yellow-spots. Hence, protein-microarray spots are observed to change from "yellow-to-red" upon antigen detection --- in a 1-step immunoassay detection scheme.

The reagents employed in the preceding examples are commercially available or can be prepared using commercially available instrumentation, methods, or reagents known in the art or whose preparation is described in the examples. It is evident from the above description and results that the subject invention is greatly superior to the presently available methods for determining the presence of a target in a biological sample. The subject invention overcomes the shortcomings of the currently used methods by allowing small quantities of antibodies to be labeled and in unlimited media while maintaining specificity and sensitivity. The examples are not intended to provide an exhaustive description of the many different embodiments of the invention. Thus, although the forgoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, those of ordinary skill in the art will realize readily that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

All publications, patents and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference.